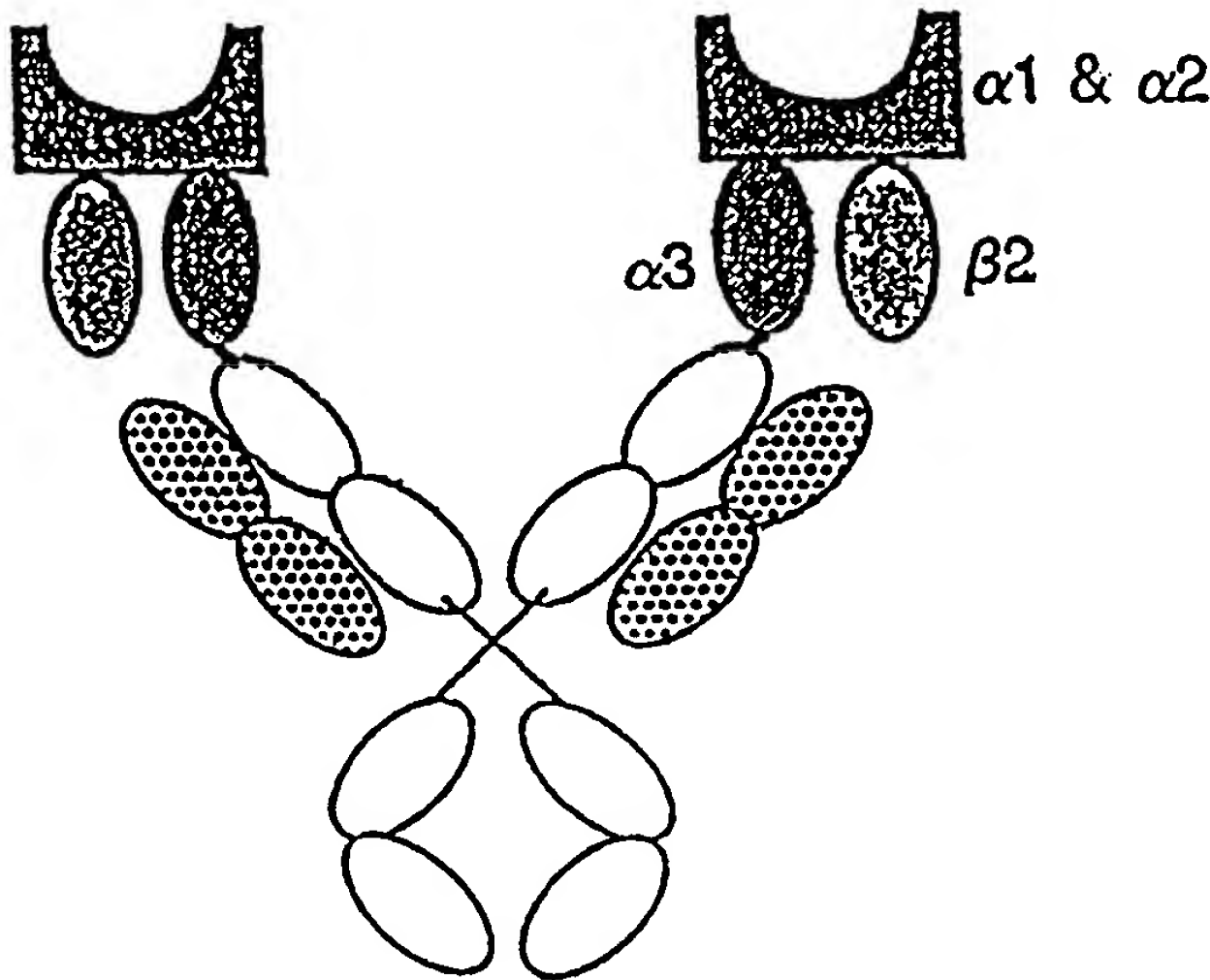


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(54) Title: USE OF MULTIVALENT CHIMERIC PEPTIDE-LOADED, MHC/IG MOLECULES TO DETECT, ACTIVATE OR SUPPRESS ANTIGEN-SPECIFIC T CELL-DEPENDENT IMMUNE RESPONSES (57) Abstract <p>To increase the effective affinity of soluble analogs of peptide/MHC molecules for their cognate ligands, divalent peptide/MHC complexes were constructed. Using a recombinant DNA strategy, DNA encoding the MHC class I was ligated to DNA coding for murine Ig heavy chain. MHC/Ig complexes were exploited to homogeneously load with peptides of interest. The results of flow cytometry demonstrated that the ^{Pep}MHC/Ig complexes bound specifically with high affinity to cells bearing their cognate receptors. ^{Pep}MHC/Ig complexes are also useful in modulating effector functions of antigen-specific T cells. These ^{Pep}MHC/Ig complexes are useful for studying TCR/MHC interactions and lymphocyte tracking and have uses as specific regulators of immune responses.</p> <div data-bbox="943 1576 1847 2316" data-label="Chemical-Block">  </div>		

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**USE OF MULTIVALENT CHIMERIC PEPTIDE-LOADED,
MHC/IG MOLECULES TO DETECT, ACTIVATE OR SUPPRESS
ANTIGEN-SPECIFIC T CELL-DEPENDENT IMMUNE RESPONSES**

5 This application is a continuation-in-part of U.S. Serial No.
08/828,712 filed March 28, 1997 and claims the benefit of co-pending
applications U.S. Serial No. 60/058,573 filed September 11, 1997 and U.S.
Serial No. 60/082,538 filed April 21, 1998, which are incorporated by
reference herein.

BACKGROUND OF THE INVENTION

10 The immune system is a defense system found in its most advanced
form in higher vertebrates. This defense mechanism provides rapid and highly
specific responses which are used to protect an organism against invasion by
pathogenic microorganisms. It is the myriad of pathogenic microorganisms
which have principally caused the evolution of the immune system to its
15 current form. In addition to protection against infectious agents, specific
immune responses are thought to be involved in surveillance against tumor
development, the pathogenesis of autoimmune diseases, and transplant tissue
rejection.

20 T cells are the major regulatory cells of the immune system. The
regulatory functions of T cells depend not only on expression of a unique T
cell receptor, but also on expression of a variety of accessory molecules and
effector functions associated with an individual T cell response (*i.e.*, a
cytotoxic response versus a response characterized by secretion of effector
molecules such as lymphokines). It is this regulatory function that often goes
25 awry in the development of autoimmune diseases, plays a large role in tissue
graft rejection, and can be important in tumor rejection.

Specificity of T cell responses is conferred by a unique set of cell
surface receptors expressed on individual lymphocytes, called clonotypic T

cell receptors (TCR). Cognate ligands of the clonotypic TCR are antigen major histocompatibility complex molecules (MHC). T cell receptors recognize antigens in the form of small antigenic peptides presented by MHC molecules on the surface of antigen presenting cells. While the interaction
5 between an antigenic peptide and an MHC is quite stable, generally displaying a high affinity (10^{-9} M) and consequent long half-life of dissociation, the interaction between a T cell receptor and a peptide/MHC complex -- the critical recognition event in triggering T cells -- is of relatively low affinity, between 10^{-4} - 10^{-6} M. As a result of this low affinity, the T cell response is
10 driven by the interaction of many T cell receptors on the surface of an individual T cell interacting with multiple antigenic peptide/MHC complexes on the surface of the antigen presenting cell.

Antigen-specific T cells play major roles in normal physiologic immune responses and in many disease states. Hyperactivation of antigen-
15 specific T cells targeted toward self antigens is the underlying basis for the majority of autoimmune diseases, including multiple sclerosis, arthritis, and diabetes. Conversely, inactivity of tumor antigen-specific T cells allows tumors to grow. Thus, activation of dormant or tolerant tumor-specific T cells has been a major goal of cancer immunotherapy. Other important
20 medical phenomena, such as rejection of transplanted organs, depend on the activity of T cells specific for alloantigens expressed by these organs. Antigen-specific suppression of undesired T cell responses could potentially eliminate organ graft rejection.

The use of soluble monovalent reagents to monitor and modulate
25 antigen-specific T cells is limited by the fact that T cell receptors interact with peptide/MHC complexes with relatively low affinities (28-30). Thus, soluble monovalent analogs of either T cell receptors or peptide/MHC complexes do not effectively regulate immune responses.

Soluble multivalent analogs of proteins involved in immune responses
30 have been made. Such analogs include CD4/Ig chimeras (41, 42), CR2/Ig chimeras (43), and class I MHC/Ig chimeras (20). The influence of valence

on ligand affinity of these analogs is variable. For example, CD4/Ig molecules do not have a higher affinity for their ligand than soluble monovalent CD4 molecules. Tetravalent peptide/MHC complexes have high affinity for specific T cell receptors. However, divalent MHC analogs do not have a high enough affinity to allow staining or regulating antigen-specific T cells. Thus, there is a need in the art for agents which can specifically induce or suppress T cells specific for antigens associated with infections, allergies, tumors, transplanted organs, and autoimmune diseases.

SUMMARY OF THE INVENTION

It is an object of the invention to provide a set of reagents which specifically and stably bind to and modulate antigen-specific T cells. This and other objects of the invention are provided by one or more of the following embodiments.

One embodiment of the invention provides a composition comprising a chimeric protein. The chimeric protein comprises an MHC molecule and an immunoglobulin chain. The chimeric protein associates to form molecular complexes comprising at least two chimeric proteins per complex. An antigenic peptide is bound to each MHC molecule. Each MHC molecule within the molecular complex is bound to an identical antigenic peptide.

Still another embodiment of the invention provides a vector encoding a chimeric protein. The chimeric protein comprises an immunoglobulin heavy chain and an MHC molecule. The immunoglobulin is not IgG-1. The immunoglobulin heavy chain is C-terminal to the MHC molecule.

Even another embodiment of the invention provides a composition comprising a chimeric protein which comprises an MHC molecule and an Ig chain. The chimeric protein associates to form molecular complexes comprising at least two chimeric proteins per complex. The immunoglobulin chain is not an IgG heavy chain.

Another embodiment of the invention provides a composition comprising a cell in which a chimeric protein is bound to the surface of the

cell. The chimeric protein comprises an MHC molecule and an immunoglobulin chain. The chimeric protein associates to form molecular complexes comprising at least two chimeric proteins per complex. An antigenic peptide is bound to each MHC molecule. Each MHC molecule within the molecular complex is bound to an identical antigenic peptide.

Still another embodiment of the invention provides a method for treating a patient suffering from an allergy. A chimeric protein which comprises an MHC molecule and an immunoglobulin chain is administered to a patient at a dose sufficient to suppress a T cell response associated with an allergy of the patient. The chimeric protein associates to form molecular complexes comprising at least two chimeric proteins per complex. An antigenic peptide is bound to each MHC molecule. Each MHC molecule within the molecular complex is bound to an identical antigenic peptide. The antigenic peptide is an antigen to which the patient has an allergic response.

Even another embodiment of the invention provides a method for treating a patient who has received or will receive an organ transplant. A chimeric protein which comprises an MHC molecule and an immunoglobulin chain is administered to the patient at a dose sufficient to suppress an immune response to the transplanted organ. The chimeric protein associates to form molecular complexes comprising at least two chimeric proteins per complex. An antigenic peptide is bound to each MHC molecule. Each MHC molecule within the molecular complex is bound to an identical antigenic peptide. The antigenic peptide is an alloantigen.

Another embodiment of the invention provides a method for treating a patient suffering from an autoimmune disease. A chimeric protein which comprises an MHC molecule and an immunoglobulin chain is administered to the patient at a dose sufficient to suppress an immune response associated with the autoimmune disease. The chimeric protein associates to form molecular complexes comprising at least two chimeric proteins per complex. An antigenic peptide is bound to each MHC molecule. Each MHC molecule within the molecular complex is bound to an identical antigenic peptide. The

antigenic peptide is one to which the patient expresses an autoimmune response.

5 Still another embodiment of the invention provides a method for treating a patient having a tumor. A chimeric protein which comprises an MHC molecule and an immunoglobulin chain is administered to the patient at a dose sufficient to induce or enhance an immune response to the tumor. The chimeric protein associates to form molecular complexes comprising at least two chimeric proteins per complex. An antigenic peptide is bound to each MHC molecule. Each MHC molecule within the molecular complex is bound to an identical antigenic peptide. The antigenic peptide is a tumor-associated peptide.

10 Another embodiment of the invention provides a method for treating a patient having an infection. A chimeric protein which comprises an MHC molecule and an immunoglobulin chain is administered to the patient at a dose sufficient to induce or enhance an immune response to the infection. The chimeric protein associates to form molecular complexes comprising at least two chimeric proteins per complex. An antigenic peptide is bound to each MHC molecule. Each MHC molecule within the molecular complex is bound to an identical antigenic peptide. The antigenic peptide is an infectious agent-associated peptide.

20 Even another embodiment of the invention provides a method of labeling antigen-specific T cells. A sample comprising antigen-specific T cells is contacted with a chimeric protein. The chimeric protein comprises an MHC molecule and an immunoglobulin chain. The chimeric protein associates to form molecular complexes comprising at least two chimeric proteins per complex. Each MHC molecule within the molecular complex is bound to an identical antigenic peptide. The antigenic peptide specifically binds to and labels the antigen-specific T cells.

25 These and other embodiments of the invention provide the art with tools and methods for modulating antigen-specific T cell immune responses.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Figure 1A, schematic of chimeric MHC/Ig molecules formed in a conformationally intact fashion at the ends of the immunoglobulin heavy chains. Figure 1B, schematic of the pA2/Ig plasmid containing the regions encoding the extracellular domains ($\alpha 1$ - $\alpha 3$) of the HLA-A2 class I MHC molecule genetically linked to the variable region of an IgG1 heavy chain gene.

Figure 2. Peptide loaded L^d /Ig molecules specifically bind to T cells expressing their cognate ligand. L^d /Ig molecules were loaded with peptides (100 μ M) overnight. Peptides used included the 2C CTL reactive peptide p2Ca, a tumor peptide specific for murine CT 26 cells, AH1, and another L^d -restricted model tumor antigen derived from β -galactosidase, β -gal. After overnight incubation, peptide/ L^d /Ig complexes were used to stain the antigen-specific T cells 2C and anti-CT 26 CTL. Viable T cells were purified using lymphocyte-M separation and reacted with 20 μ g/ml of the peptide/ L^d /Ig complex for 1 hour at RT. Cells were then washed and stained with goat anti-mouse PE and analyzed by flow cytometry. Representative histograms are shown. Figure 2A. 2C CTL were reactive with $p2Ca$ - L^d /Ig complexes but not with $AH1$ - L^d /Ig complexes. Figure 2B. CT 26 specific T cells were only reactive with $AH1$ - L^d /Ig but not with either $p2Ca$ - L^d /Ig or β -gal- L^d /Ig complexes.

Figure 3. Schematic of K^b /IgG loading scheme.

Figure 4. Peptide stripping and reloading enhances effective peptide loading of K^b /Ig molecules. K^b /Ig molecules were either "passively" or "actively" loaded with peptides (100 μ M) as described. Peptides used included the 2C CTL reactive peptide SIY. A control peptide included another K^b -binding peptide, VSV. Varying dilutions of P^{sp} MHC/Ig complexes were used to stain 2C specific T cells. Viable T cells were purified using lymphocyte-M separation and reacted with 20 μ g/ml of the peptide/ L^d /Ig complex for 1 hour at 4°C. Cells were then washed and stained with goat anti-mouse PE and analyzed by flow cytometry. Representative histograms are shown.

are shown.

Figure 5. Peptide loaded MHC/Ig molecules bind T cells with high affinity. L^d /Ig or K^b /Ig molecules were loaded with peptides (100 μ M) as described. Peptides used included the 2C CTL reactive H-2L^d-restricted peptides QL9, and p2Ca, and the 2C CTL reactive H-2k^b restricted peptide SIY. Control peptides included another L^d-binding peptide MCMV and a K^b-binding peptide VSV. Varying concentration of peptide/MHC/Ig complexes were used to stain 2C specific T cells as previously described. Representative histograms were analyzed and the data are presented as mean channel fluorescence values.

Figure 6. SEC Analysis of peptide loaded MHC/Ig molecules. Approximately 50 μ g of 35 S-K^b/Ig or 35 S-L^d/Ig molecules were loaded onto a size exclusion chromatography column (Superdex 200, Pharmacia Biotech). The column was run with a PBS running buffer at 0.4 ml/min. on a BioLogic chromatography system (BioRad, Hercules, CA.) with fractions collected every 0.4 ml. O.D. 280 are plotted for both the chimeric proteins.

Figure 7. Staining of 2C CTL is due to the monomeric fraction of 35 S-K^b/Ig. K^b/Ig molecules were loaded with SIY peptide, 100 μ M final concentration, as described. Viable T cells were purified using lymphocyte-M separation and reacted with either 1/5 or 1/50 dilution of each fraction collected by a preparative SEC analysis of 35 S-K^b/Ig (500 μ g), as described above. Cells were incubated with each fraction collected from the SEC analysis for 1 hour at 4°C. Cells were then washed and stained with goat anti-mouse PE and analyzed by flow cytometry. Data shown are the mean channel fluorescence obtained from representative samples. The experiments were repeated at least twice. Representative data are presented.

Figure 8. Detection of AH1-specific T cells from spleen 14 days after vaccination with CT26-GM.

Figure 9. 51 Cr-MHC/Ig complexes specifically inhibit peptide-dependent lysis of target cells by 2C CTL. Chromium labeled T2K^{bm3} target cells were preincubated at 25°C for 2.5 h with dEV8 peptide (10 nM). Concurrently,

2C T cells (3×10^4 /well) were incubated with the indicated ligand at various concentrations for 1.5 h in V-bottom wells. After incubations, unwashed targets (3×10^3 per well) were added to the effectors/ligands, and the plates were subsequently incubated 4 h at 37°C before harvesting and counting.

5 Standard percent specific lysis was determined.

Figure 10. ^{51}Cr MHC/Ig complexes specifically inhibit proliferation of 2C CTL by allogeneic target cells. 2C T cells were incubated with various concentrations of ^{51}Cr K^b/Ig for 2.5 hours at 37°C. Irradiated (3000 rads) freshly isolated BALB/c splenocytes (expressing H-2 L^d) were added to the responders cells ligands at 2.5×10^5 /well. Following a 60 h incubation, cells were pulsed with [^3H]thymidine and incubated for an additional 18 h before being harvested and counted.

Figure 11. Peptide loaded HLA-A2/Ig specifically stains HTLV-1 Tax11-19 or HIV p17 Gag77-85-specific T cell clones. Figure 11A. FACS analysis was carried out using A6 cells (22), specific for the immunodominant HLA-A2 restricted Tax epitope Tax11-19. HTLV-1 Tax11-19 loaded HLA-A2/IgG stably bound on the cell surface and was detected using PE-labeled goat-anti mouse Ig (heavy line). Gag-A2/Ig was used as an irrelevant control (thin line) as well as no HLA-A2/Ig, unloaded HLA-A2/Ig and M1-loaded HLA-A2/Ig (dotted and stripped lines overlapping). Figure 11B. T cells specific for the HLA-A2-restricted HIV p17 Gag 77-85 epitope were stained to demonstrate the peptide-specificity of peptide-loaded HLA-A2/Ig. Gag77-85 loaded HLA-A2/IgG was stably bound on the cell surface and detected using PE-labeled goat-anti mouse Ig (heavy line). HTLV-1 Tax11-19 loaded HLA-A2/Ig as well as no peptide loaded, M1-loaded HLA-A2/Ig, and no HLA-A2/Ig were used as an irrelevant control and stained virtually identical (overlapping thin, dotted and stripped lines).

Figure 12. Peptide loaded HLA-A2/Ig can be used to visualize antigen-specific CD8⁺ T cells in peripheral blood. Two-color flow cytometry was performed using Tax-A2/Ig or Gag-A2/Ig and mouse anti-human

CD8-FITC. PE-labeled goat anti-mouse IgG1 monoclonal antibody was used to detect the peptide-A2/Ig complex. Frozen PBL from an HAM/TSP patient (donor H1 see Table 1) were examined. Anti-human CD8-FITC and Tax-A2/Ig positively stained 4.03% of the PBL from the HAM/TSP patient. This represents 10.49% of all CD8⁺ cells. No significant signal was seen using Gag-A2/Ig (< 0.1%). PBL from an HLA-A2⁺ HIV-1 infected individual showed 3% Gag-A2/Ig positive CD8⁺ T lymphocytes.

Figure 13. HAM/TSP patients carry high numbers of HTLV-1 Tax11-19-specific CD8⁺ cells in their PBL. The percent of HTLV-1 Tax11-19-specific, CD8⁺ T cells from HLA-A2⁺ HAM/TSP patients, HTLV-1 infected HLA-A2⁺ carriers, HLA-A2⁻ HAM/TSP patients, and healthy individuals are presented. Between 0.64 and 10.25% of the CD8⁺ cells from HAM/TSP patients are specific for the HTLV-1 Tax11-19 peptide. One of the analyzed HTLV-1 infected donors had 0.33% Tax-specific CD8⁺ cells, whereas no significant number of Tax-specific CD8⁺ cells were found in non-HLA-A2 HAM/TSP donors or HLA-A2⁺ healthy individuals. Positive samples were at least stained twice.

Figure 14. HTLV-1 Tax11-19 specific CD8⁺ T cells accumulate in cerebrospinal fluid. Cerebrospinal fluid and peripheral blood were obtained from an HAM/TSP patient (H1) the same day and analyzed using Tax-A2/Ig. 9 x 10⁴ cells were obtained from cerebrospinal fluid and stained using Tax/A2-Ig and Gag/A2-Ig together with mouse anti-human CD8-FITC, as described in Figure 13. Freshly obtained, Ficoll purified PBL were analyzed in parallel. Figure 14A. Of the CD8⁺ T cells in cerebrospinal fluid, 23.7% were Tax-specific, and only 9.4% of the CD8⁺ T cells from peripheral blood were Tax-specific. No significant signal was seen using Gag-A2/Ig (< 0.1%). Figure 14B. CD4/CD8 analysis showed that the ratio of CD4/CD8 was 1.1 in the CSF and 1.5 in the PBL, due to a higher number of CD8⁺ T cells in CSF.

Figure 15. Tax-specific CD8⁺ cells from HAM/TSP patients express the cell surface activation marker HLA-DR. Using three-color flow cytometry, Tax-specific CD8⁺ cells were gated and analyzed for HLA-DR

expression using mouse anti-human HLA-DR-FITC (solid line) and an isotype matched irrelevant mouse IgG2a-FITC control (dotted line). Figures 15A and 15B. In 2 different symptomatic HAM/TSP patients, 41-50% of the Tax-specific CD8⁺ T cells stained positive for HLA-DR expression. Figure 15C. In contrast, only 21% of the Tax-specific CD8⁺ T cells from patient H6, who has a very mild form of the disease, stained positive. Figure 15D shows a non-stimulated staining control. Figure 15E shows control staining from PHA-stimulated CD8⁺ PBL from a healthy donor.

Figure 16. Tax-specific CD8⁺ cells from HAM/TSP patients express intracellular IFN- γ and TNF- α . Using three-color flow cytometry, Tax-specific CD8⁺ cells were gated and analyzed for intracellular cytokine expression. Figure 16A. Using mouse anti-human IFN- γ -PE, mouse anti-human TNF- α -PE, and an isotype matched irrelevant mouse IgG1-PE control, 28% of the Tax-specific CD8⁺ cells from H1 expressed intracellular IFN- γ , and 29% expressed TNF- α . Figure 16B. Only 8% of the Tax-specific CD8⁺ cells from H6 expressed intracellular IFN- γ and TNF- α .

Figure 17. High levels of HTLV-1 Tax-specific CD8⁺ T lymphocytes persist during HAM/TSP. PBL samples obtained between 1989 and 1998 from patient H1 were analyzed for Tax-specific, CD8⁺ cells. Between 7.24 and 13.87% specific CD8⁺ T cells were detected in the frozen PBL samples.

Figure 18. Structure of HLA-A2 complexed with Tax 11-19 peptide. Two views of the 3-D structure of HLA-A2 heavy chain (green), β 2M (purple) and tax 11-19 antigenic peptide (red) are shown. The carboxyl terminal, position 9, of tax 11-19 peptide and amino terminal position 1 of the β 2M polypeptides are represented as space filling diagrams while the rest of the chains are shown as ribbon diagrams. Oxygen groups in the space filled amino acid residues are represented as red balls while the nitrogen groups are seen as light blue ones. The distance between the amino terminus of the β 2M molecule and position 9 of the peptide was found to be 20.49 Angstroms. Open access of the region between the ends of the peptide and β 2M molecule can be seen best on the side view of the complex (left-hand side of Figure 18).

the peptide for the class I complex secondary to modification of the carboxyl terminal tax peptide, position 9. Anchoring of the peptide to the MHC molecule is achieved in part through bonds to the carboxylterminal oxygen that will be unavailable in the tethered peptide- β 2M complex. This decreased affinity may be compensated for by stabilization with β 2M.

Figure 19. Schematic oligo design used to generate peptides tethered to 2M.

Figure 20. Specific interaction of peptide-tethered tax- β 2M HLA-A2/Ig complexes with HLA-A2 restricted CTL clones analyzed by flow cytometry. Mean channel fluorescence (MCF) of A6 cells stained with peptide tethered tax- β 2M HLA-A2/Ig complexes was approximately 4000, MCF of A6 cells stained using no chimeric protein was only 10 and almost identical to staining controls with the control Gag-A2/Ig complex, unloaded HLA-A2/Ig. The level of staining with tax- β 2M was similar to that obtained with an anti-V β 13.1 specific mAb, indicating that all the relevant sites were saturated using tax- β 2M HLA-A2/Ig. Thus, peptide tethering to β 2M is an efficient way to present peptides in the context of MHC/Ig molecules.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides soluble, high affinity, multivalent analogs of MHC molecules. These reagents can be used diagnostically, to detect antigen-specific T cells, or for therapy, either to activate or suppress antigen-specific T cells *in vitro* and *in vivo*. Therefore, these reagents can be used in selective immune-based therapy of infections, allergies, autoimmune diseases, cancer, and organ transplant rejection.

The reagents of the invention express a single peptide/MHC complex in a multivalent array. The multivalent array is formed using an immunoglobulin backbone as a molecular scaffold for presentation of an antigenic peptide/class I complex. These arrays can be made by producing chimeric genes in which an MHC gene is linked to the 5' end of an immunoglobulin heavy chain, as described below. When these constructs are

expressed, for example, in baculovirus or hybridoma cells, they produce chimeric molecules which are multivalent, based on the valence of the molecular scaffold, the immunoglobulin molecule.

5 The chimeric MHC/Ig molecules are formed in a conformationally intact fashion at the ends of the immunoglobulin heavy chains (see Figure 1A for a schematic representation). When homogeneously loaded with antigenic peptides, MHC/Ig chimeras (^PMHC/Ig molecules) have sufficiently high affinity to produce stable binding and can modulate antigen-specific T cells.

10 These constructs have a number of other useful features. For example, they are extremely stable and easy to produce, based on the stability and secretion efficiency provided by the immunoglobulin backbone. Further, by altering the Fc portion of the immunoglobulin, different biological functions can be provided to the molecule based on biological functions afforded by the Fc portion. Substitution of the Fc portion of one type of
15 immunoglobulin gene for another is within the skill of the art.

This class of reagents represents a new tool for understanding a variety of immunologic diseases. The observation that a divalent MHC/Ig chimeric protein stably binds to its cognate T cell receptor is surprising since the monovalent peptide-MHC complex dissociates rapidly from complexes
20 with TCR with a half life of less than 1 minute (27) and the affinity of the T cell receptor for peptide-MHC complexes is relatively low ($\sim 10^{-5}$ μ M) (28-30). Stable binding of the dimeric HLA-A2/Ig complex to cognate T cells is likely based on two features related to the immunoglobulin scaffold. First, the multivalent nature of the complex provides an increase in avidity.
25 Second, the unique flexibility of the immunoglobulin hinge region likely promotes maximal avidity enhancement with our divalent construct.

Chimeric proteins of the invention comprise an immunoglobulin heavy chain and an MHC molecule. The immunoglobulin heavy chain can be the heavy chain of an IgM, IgD, IgG1, IgG3, IgG2 β , IgG2 α , IgE, or IgA.
30 Preferably, an IgG heavy chain is used to form divalent chimeric proteins of the invention. If multivalent chimeric proteins are desired, IgM or IgA heavy

chains can be used to provide pentavalent or tetravalent chimeras, respectively. Chimeric proteins with other valencies can also be constructed, using multiple immunoglobulin heavy chains.

5 The immunoglobulin heavy chain is fused to a polypeptide chain of an MHC molecule. Methods of making fusion proteins, either recombinantly or by covalently linking two protein segments, are well known. Preferably, fusion proteins are expressed recombinantly, as products of expression constructs. Expression constructs of the invention comprise a polynucleotide or vector which encodes a fusion protein of the invention in which an
10 immunoglobulin heavy chain is C-terminal to an MHC molecule.

Expression vectors of the invention can be introduced into host cells using any technique known in the art. These techniques include transferrin-polycation-mediated DNA transfer, transfection with naked or encapsulated nucleic acids, liposome-mediated cellular fusion, intracellular transportation of
15 DNA-coated latex beads, protoplast fusion, viral infection, electroporation, and calcium phosphate-mediated transfection.

Host cells comprising expression vectors of the invention can be prokaryotic or eukaryotic. Bacterial systems for expressing fusion proteins of the invention include those described in Chang *et al.*, *Nature* (1978) 275: 615,
20 Goeddel *et al.*, *Nature* (1979) 281: 544, Goeddel *et al.*, *Nucleic Acids Res.* (1980) 8: 4057, EP 36,776, U.S. 4,551,433, deBoer *et al.*, *Proc. Natl. Acad. Sci. USA* (1983) 80: 21-25, and Siebenlist *et al.*, *Cell* (1980) 20: 269.

Expression systems in yeast include those described in Hinnen *et al.*, *Proc. Natl. Acad. Sci. USA* (1978) 75: 1929; Ito *et al.*, *J. Bacteriol.* (1983) 153: 163; Kurtz *et al.*, *Mol. Cell. Biol.* (1986) 6: 142; Kunze *et al.*, *J. Basic Microbiol.* (1985) 25: 141; Gleeson *et al.*, *J. Gen. Microbiol.* (1986) 132: 3459, Roggenkamp *et al.*, *Mol. Gen. Genet.* (1986) 202 :302) Das *et al.*, *J. Bacteriol.* (1984) 158: 1165; De Louvencourt *et al.*, *J. Bacteriol.* (1983) 154: 737, Van den Berg *et al.*, *Bio/Technology* (1990) 8: 135; Kunze *et al.*, *J. Basic Microbiol.* (1985) 25: 141; Cregg *et al.*, *Mol. Cell. Biol.* (1985) 5: 3376, U.S. 4,837,148, US 4,929,555; Beach and Nurse, *Nature* (1981) 300:
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706; Davidow *et al.*, *Curr. Genet.* (1985) 10: 380, Gaillardin *et al.*, *Curr. Genet.* (1985) 10: 49, Ballance *et al.*, *Biochem. Biophys. Res. Commun.* (1983) 112: 284-289; Tilburn *et al.*, *Gene* (1983) 26: 205-221, Yelton *et al.*, *Proc. Natl. Acad. Sci. USA* (1984) 81: 1470-1474, Kelly and Hynes, *EMBO J.* (1985) 4: 475479; EP 244,234, and WO 91/00357.

Expression of fusion proteins of the invention in insects can be carried out as described in U.S. 4,745,051, Friesen *et al.* (1986) "The Regulation of Baculovirus Gene Expression" in: THE MOLECULAR BIOLOGY OF BACULOVIRUSES (W. Doerfler, ed.), EP 127,839, EP 155,476, and Vlak *et al.*, *J. Gen. Virol.* (1988) 69: 765-776, Miller *et al.*, *Ann. Rev. Microbiol.* (1988) 42: 177, Carbonell *et al.*, *Gene* (1988) 73: 409, Maeda *et al.*, *Nature* (1985) 315: 592-594, Lebacq-Verheyden *et al.*, *Mol. Cell. Biol.* (1988) 8: 3129; Smith *et al.*, *Proc. Natl. Acad. Sci. USA* (1985) 82: 8404, Miyajima *et al.*, *Gene* (1987) 58: 273; and Martin *et al.*, *DNA* (1988) 7:99. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts are described in Luckow *et al.*, *Bio/Technology* (1988) 6: 47-55, Miller *et al.*, in GENETIC ENGINEERING (Setlow, J.K. *et al.* eds.), Vol. 8 (Plenum Publishing, 1986), pp. 277-279, and Maeda *et al.*, *Nature*, (1985) 315: 592-594.

Expression of fusion proteins of the invention in mammalian cells can be achieved as described in Dijkema *et al.*, *EMBO J.* (1985) 4: 761, Gorman *et al.*, *Proc. Natl. Acad. Sci. USA* (1982b) 79: 6777, Boshart *et al.*, *Cell* (1985) 41: 521 and U.S. 4,399,216. Other features of mammalian expression can be facilitated as described in Ham and Wallace, *Meth. Enz.* (1979) 58: 44, Barnes and Sato, *Anal. Biochem.* (1980) 102: 255, U.S. 4,767,704, US 4,657,866, US 4,927,762, US 4,560,655, WO 90/103430, WO 87/00195, and U.S. RE 30,985.

Each MHC molecule within a molecular complex is bound to an identical antigenic peptide. Any peptide capable of inducing an immune response can be bound to the MHC molecule, including peptides which cause allergic or autoimmune responses, alloantigens, tumor-associated peptides,

and peptides of infectious agents, such as bacteria, viruses, or fungi.

Antigenic peptides can be bound to the antigen binding site of the MHC molecule or to the amino-terminus of either an MHC class II β chain or a β_2 microglobulin. Binding, or "loading" of the antigenic peptide to the chimeric protein can be carried out actively or passively, as described in Example 2, below. The antigenic peptide can be covalently bound to the chimeric protein.

Optionally, a peptide tether can be used to link an antigenic peptide to β_2 microglobulin. Crystallographic analyses of multiple class I MHC molecules indicate that the amino terminus of β_2 M is very close, approximately 20.5 Å away, from the carboxyl terminus of the antigenic peptide resident in the MHC peptide binding groove (see Figure 18). Thus, using a relatively short linker sequence, approximately 13 amino acids in length, one can tether a peptide to the amino terminus of β_2 M. If the sequence is appropriate, that peptide will bind to the MHC binding groove (see Example 17, below).

Molecular complexes of the invention can be used diagnostically, to label antigen-specific cells *in vitro* or *in vivo*. A sample comprising antigen-specific T cells can be contacted with a molecular complex in which each MHC molecule is bound to an identical antigenic peptide. The sample can be, for example, peripheral blood, lymphatic fluid, lymph nodes, spleen, thymus, or bone marrow.

The antigenic peptide specifically binds to the antigen-specific T cells and labels them with the antigenic peptide-loaded complex. Antigenic peptide/MHC complexes can be, but need not be, conjugated to a reporter group, such as a radiolabel or fluorescent label, to facilitate detection. The molecular complex can be in solution or can be affixed to a solid substrate, such as a glass or plastic slide or tissue culture plate or latex, polyvinylchloride, or polystyrene beads.

Antigen-specific T cells which are bound to the antigenic peptides can be separated from cells which are not bound. Any method known in the art

can be used to achieve this separation, including plasmapheresis, flow cytometry, or differential centrifugation. Antigen-specific T cells which have been isolated from a patient can be treated with a reagent, such as a cytokine, a chemotherapeutic agent, or an antibody, and reinfused into the patient to provide a therapeutic effect. Optionally, the number of antigen-specific T cells which are bound to the antigenic peptides can be quantitated or counted, for example by flow cytometry.

Molecular complexes of the invention can also be used to activate or inhibit antigen-specific T cells. It is possible to conjugate toxin molecules, such as ricin or *Pseudomonas* toxin, to molecular complexes of the invention. Similarly, molecular complexes can be conjugated to molecules which stimulate an immune response, such as lymphokines or other effector molecules. Doses of the molecular complex can be modified to either activate or inhibit antigen-specific T cells.

A sample which comprises antigen-specific T cells can be contacted, *in vivo* or *in vitro*, with molecular complexes in which each MHC molecule is bound to an antigenic peptide. The antigenic peptide specifically binds to and activates or inhibits the antigen-specific T cells. For example, cytokine release from specific T cell subsets can be stimulated or suppressed. Hewitt *et al.*, *J. Exp. Med.* 175:1493 (1992); Choi *et al.*, *Proc. Natl. Acad. Sci.* 86:8941 (1989); Kappler *et al.*, *Science* 244:811 (1989); Minasi *et al.*, *J. Exp. Med.* 177:1451 (1993); Sundstedt *et al.*, *Immunology* 82:117 (1994); and White *et al.*, *Cell* 56:27 (1989).

Molecular complexes of the invention can be used therapeutically, to inhibit or stimulate immune responses. For example, molecular complexes comprising antigenic peptides to which a patient has an allergic response can be administered to the patient in order to treat an allergy. The molecular complexes are administered to the patient at a dose sufficient to suppress or reduce a T cell response associated with the allergy.

Similarly, a patient who has received or will receive an organ transplant can be treated with molecular complexes of the invention.

Molecular complexes in which each ligand binding site is bound to an alloantigen can be administered to a patient at a dose sufficient to suppress or reduce an immune response to the organ transplant. Alloantigens include the HLA antigens, including class I and class II MHC molecules, and minor histocompatibility antigens such as the ABO blood group antigens, autoantigens on T and B cells, and monocyte/endothelial cell antigens.

Autoimmune diseases, such as Goodpasture's syndrome, multiple sclerosis, Graves' disease, myasthenia gravis, systemic lupus erythematosus, insulin-dependent diabetes melitis, rheumatoid arthritis, pemphigus vulgaris, Addison's disease, dermatitis herpetiformis, celiac disease, and Hashimoto's thyroiditis, can be similarly treated. A patient who suffers from an autoimmune disease can be treated with molecular complexes of the invention in which each ligand binding site is bound to an antigenic peptide to which the patient expresses an autoimmune response. The molecular complexes are administered to the patient at a dose sufficient to suppress or reduce the autoimmune response.

Immune responses of a patient can also be induced or enhanced using molecular complexes of the invention. Molecular complexes in which each ligand binding site is bound to a peptide expressed by a tumor can be used to treat the tumor. The peptide can be a tumor-specific peptide, such as EGFRvIII, Ras, or p185^{HER2}, or can be a peptide which is expressed both by the tumor and by the corresponding normal tissue. Similarly, molecular complexes in which each ligand binding site is bound to a peptide of an infectious agent, such as a protein of a bacterium or virus, can be used to treat infections. In each case, the appropriate molecular complexes are administered to the patient at a dose sufficient to induce or enhance an immune response to the tumor or the infection.

Molecular complexes of the invention can be bound to the surface of a cell, such as a dendritic cell. A population of molecular complexes in which all ligand binding sites are bound to identical antigenic peptides can also be bound to the cell. Binding can be accomplished by providing the fusion

protein of the molecular complex with an amino acid sequence which will anchor it to the cell membrane and expressing the fusion protein in the cell or can be accomplished chemically, as is known in the art.

5 Compositions comprising molecular complexes of the invention can comprise a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well known to those in the art. Such carriers include, but are not limited to, large, slowly metabolized macromolecules, such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and inactive virus particles. Pharmaceutically
10 acceptable salts can also be used in compositions of the invention, for example, mineral salts such as hydrochlorides, hydrobromides, phosphates, or sulfates, as well as salts of organic acids such as acetates, propionates, malonates, or benzoates. Compositions of the invention can also contain liquids, such as water, saline, glycerol, and ethanol, as well as substances such
15 as wetting agents, emulsifying agents, or pH buffering agents. Liposomes, such as those described in U.S. 5,422,120, WO 95/13796, WO 91/14445, or EP 524,968 B1, can also be used as a carrier for a composition of the invention.

 The particular dosages of divalent and multivalent molecular
20 complexes employed for a particular method of treatment will vary according to the condition being treated, the binding affinity of the particular reagent for its target, the extent of disease progression, etc. However, the dosage of molecular complexes will generally fall in the range of 1 pg/kg to 100 mg/kg of body weight per day. Where the active ingredient of the pharmaceutical
25 composition is a polynucleotide encoding fusion proteins of a molecular complex, dosage will generally range from 1 nM to 50 μ M per kg of body weight.

 The amounts of each active agent included in the compositions employed in the examples described herein provide general guidance of the
30 range of each component to be utilized by the practitioner upon optimizing the method of the present invention for practice either *in vitro* or *in vivo*.

Moreover, such ranges by no means preclude use of a higher or lower amount of a component, as might be warranted in a particular application. For example, the actual dose and schedule may vary depending on whether the compositions are administered in combination with other pharmaceutical compositions, or depending on individual differences in pharmacokinetics, drug disposition, and metabolism. Similarly, amounts may vary for *in vitro* applications depending on the particular cell line utilized, *e.g.*, the ability of the plasmid employed to replicate in that cell line. For example, the amount of nucleic acid to be added per cell or treatment will likely vary with the length and stability of the nucleic acid, as well as the nature of the sequence, and may be altered due to factors not inherent to the method of the present invention, *e.g.*, the cost associated with synthesis, for instance. One skilled in the art can easily make any necessary adjustments in accordance with the necessities of the particular situation.

A sufficient dose of the composition for a particular use is that which will produce the desired effect in a host. This effect can be monitored using several end-points known to those skilled in the art. For example, one desired effect might comprise effective nucleic acid transfer to a host cell. Such transfer could be monitored in terms of a therapeutic effect, *e.g.*, alleviation of some symptom associated with the disease being treated, or further evidence of the transferred gene or expression of the gene within the host, *e.g.*, using PCR, Northern or Southern hybridization techniques, or transcription assays to detect the nucleic acid in host cells, or using immunoblot analysis, antibody-mediated detection, or the assays described in the examples below, to detect protein or polypeptide encoded by the transferred nucleic acid, or impacted level or function due to such transfer.

The examples describe the use of chimeric molecules of the invention when loaded with antigenic peptides in the detection and regulation of antigen-specific T cell responses. In these examples, MHC class I molecules are used to target CD8 cells. However, MHC class II/Ig chimeras can also be produced by linking the α and β chains of MHC class II molecules to the ends

of the heavy and light chains of the immunoglobulin, respectively.

Quantitation of HTLV-1 Tax11-19-specific T cells in peripheral blood with peptide-loaded HLA-A2/Ig demonstrates that previous precursor frequency analysis of Tax-specific, CD8⁺, CTL by LDA may have significantly underestimated the actual number of Tax-specific T cells (12). Using reagents of the invention, we were able to show that the number of Tax-specific CD8⁺ T cells assessed by Tax-A2/Ig staining was roughly 10 to 30 fold higher than previously estimated by LDA (12). The accuracy of LDA is probably compromised by the fact that it depends on the ability to expand individual cells *in vitro* to numbers large enough to be detected by functional tests such as chromium release. (31-34). The frequencies determined by direct staining with Tax-A2/Ig are not due to non-specific binding. Tax-A2/Ig positively staining CD8⁺ cells were virtually undetectable in HTLV-1⁻ individuals, in HLA-A2⁻ patients with HAM/TSP and in control HLA-A2 patient infected with a different retrovirus, HIV, where a significant number of cells were specific for p17 Gag 77-85 when stained with p17 Gag 77-85 loaded A2/Ig.

In addition to using peptide-loaded HLA-A2/Ig to analyze antigen-specific T cell populations, the immunoglobulin scaffold of the HLA-A2 Ig chimera provides a variety of other options, including targeting of antigen-specific T cells *in vivo*. The diversity of *in vivo* biological effects mediated by the different Fc regions which can be inserted onto this molecule allow for its application in targeting antigen-specific T cells *in vivo*, either for amplification of antigen-specific T cells as a vaccine or for elimination of pathogenic T cells in diseases such HAM/TSP as well as classical autoimmune diseases.

The high number of HTLV-1 Tax11-19-specific CD8⁺ T cells in HLA-A2⁺ HAM/TSP patients suggests the HTLV-1 Tax11-19 may represent the immunodominant epitope in this pathogenic immune response. Thus, soluble divalent MHC/Ig can also have therapeutic application in targeting and eliminating HTLV-1-specific CD8⁺ T lymphocytes in HAM/TSP.

In the examples presented below the molecules are divalent, based on a molecular scaffold using an IgG molecule, but could also be made multivalent using an IgM for the immunoglobulin portion or using other immunoglobulin molecules as a backbone. The same genetic constructs can be used, substituting known genes encoding other Ig molecules for the IgG-1 exemplified below. Such substitution is well within the skill of the art.

The examples are provided for exemplification purposes only and are not intended to limit the scope of the invention described in broad terms above.

EXAMPLE 1

This example demonstrates staining of antigen-specific T cells by peptide loaded MHC/Ig chimeras.

The increased affinity of peptide-loaded MHC/Ig molecules allows for stable binding to cognate T cells (Figure 2). Antigen-specific T cells are specifically stained with MHC class I/IgG reagents loaded with the defined antigenic peptides. Using soluble divalent murine class I, H-2 L^d/Ig loaded with either p2Ca, a peptide reactive with 2C cytotoxic T lymphocytes (CTL), or AH-1, a peptide-specific for a tumor CTL line that recognizes the murine tumor CT26, one can label antigen-specific CTL. As shown in Figure 2A, 2C CTL were reactive with p2Ca^d/Ig complexes but not with AH1^d/Ig complexes. CT 26 specific T cells were only reactive with AH1^d/Ig but not with either p2Ca^d/Ig (not shown) or β-gal^d/Ig complexes (Figure 2B). In contrast to these results, tetravalent peptide/MHC complexes were previously found to be required for staining when the MHC was coordinated around avidin.

EXAMPLE 2

This example demonstrates passive versus active loading of the chimeras.

A critical feature of these chimeric compounds is the ability to load

each of the MHC portions with a single well-defined peptide representing the peptide antigen of interest. To effectively study the influence of divalent MHC on antigen-specific responses, parameters for peptide reconstitution of divalent MHC molecules were established. These parameters vary between the different MHC/Ig chimeras.

A variety of different approaches were analyzed to optimize peptide loading of the chimeric MHC/Ig molecules. Optimal loading of H-2 K^b/Ig was found to entail alkaline stripping, rapid neutralization, and slow refolding (see Figure 3 for a schematic). This protocol is referred to herein as "actively" loading ^{Pep}MHC/Ig complexes. "Passively" loading ^{Pep}MHC/Ig complexes is accomplished simply by incubating peptides of interest with the MHC/Ig complex over 48 hours.

When tested as reagents for staining specific T cells, "actively" loaded ^{SIY}K^b/Ig molecules were significantly better than "passively" loaded ^{SIY}K^b/Ig. Staining of antigen-specific T cells was seen at concentrations as low as 0.1 nM with "actively" loaded ^{SIY}K^b/Ig, mean channel fluorescence (MCF) of cells stained with 0.1 nM of an "actively" loaded ^{SIY}K^b/Ig was 53, compared to MCF of 42 for K^b/Ig that was "actively" loaded with a control peptide, VSV.

Staining of antigen-specific T cells required approximately 10 nM of "passively" loaded ^{SIY}K^b/Ig. Half maximal staining of antigen-specific 2C T cells was seen with 1 nM of "actively" loaded ^{SIY}K^b/Ig, but required approximately 10 nM of "passively" loaded ^{SIY}K^b/Ig. Thus, the "actively" loaded ^{Pep}K^b/Ig complexes are approximately 10-100 fold more effective than "passively" loaded ^{Pep}MHC/Ig complex in staining peptide specific T cells.

EXAMPLE 3

This example demonstrates the sensitivity of ^{Pep}MHC/Ig complexes.

The utility of these reagents is not indicated solely by their specificity but also their sensitivity. Low concentrations of ^{Pep}MHC/Ig complexes detect antigen-specific T cells. Nanomolar concentrations of peptide-loaded MHC/Ig complexes can be used to stain 2C T cells. When analyzed with

either $Q^{19}L^d/Ig$ or $S^{1Y}K^b/Ig$ complexes, half maximal stabilization can be seen at approximately 1 nM. In contrast, control peptide loaded MHC/Ig complexes, $V^{SV}K^b/Ig$ or $M^{CMV}L^d/Ig$, do not specifically interact with T cells.

Thus, peptide loaded MHC/Ig molecules are very sensitive reagents in analyzing antigen-specific T cells.

EXAMPLE 4

This example demonstrates that divalent MHC/Ig contains the active fraction of MHC/Ig.

In light of the prior reported data indicating that tetravalent MHC was required for staining of class I restricted, antigen-specific T cells, we entertained the possibility that T cell staining with the $P^{CP}MHC/Ig$ complexes was due to staining by aggregated fractions of the $P^{CP}MHC/Ig$ complexes. To address this question, we fractionated the $P^{CP}MHC/Ig$ molecules by size exclusion chromatography (SEC) and analyzed each fraction for the ability to stain antigen-specific T cells.

Initial SEC experiments indicated a variety of protein peaks in preparations of $P^{CP}MHC/Ig$ molecules (Figure 6). Both peptide loaded L^d/IgG and K^b/IgG had significant amounts of aggregated material (Peak A), with protein molecular weight $> 6.6 \times 10^5$. Interestingly, the different chimeric proteins had different amounts of protein found in the aggregates.

A second peak with a molecular weight of 2.6×10^5 K^b was found and represented the monomeric divalent $P^{CP}MHC/Ig$ peak (peak B). In the divalent L^d/Ig protein this was the prominent protein peak. This peak represented a significant but smaller fraction of protein associated with $S^{1Y}K^b/Ig$. In addition to aggregates and monomeric protein complexes, additional breakdown products were found in certain $P^{CP}MHC/Ig$ preparations. Peak C probably represents the breakdown of the chimeric molecule to IgG fraction and D and E represent smaller breakdown products that were also evident in the $S^{1Y}K^b/Ig$ preparation.

To determine which fraction was active in the flow cytometry assays,

^{125}I MHC/Ig complexes were preparatively fractionated by SEC and tested for their ability to bind CTL. The dominant T cell binding activity was associated with the monomeric divalent MHC/Ig, protein peak B, and not the aggregated protein fraction (Figure 7). While a limited amount of staining was observed with the aggregated material, protein peak A, this was relatively minimal and titered out rapidly. In contrast the monomeric peak containing the peptide loaded-divalent MHC/Ig fraction contained the overwhelming majority of the ^{125}I MHC/Ig molecules responsible for CTL staining (Figure 7).

Thus, using Ig as a molecular scaffold we have generated a high affinity divalent ^{125}I MHC/Ig analog that can be used to stain antigen-specific T cells.

EXAMPLE 5

This example demonstrates detection of antigen-specific T cells from immunized animals.

Current approaches to monitoring antigen-specific T cells have distinct limitations associated with each approach. Monitoring of T cells requires use of broadly reactive monoclonal antibodies, such as anti-V β specific monoclonal antibodies, development of a highly specific anti-clonotypic monoclonal antibody, or use of an indirect cellular based assay. While highly specific, anti-clonotypic monoclonal antibodies are relatively rare and difficult to generate. In addition, while anti-clonotypic monoclonal antibodies will recognize an antigen-specific T cell expressing a specific TCR α/β complex, they do not recognize other antigen-specific T cells that see the same antigen/MHC complex using a different α/β TCR. Cell-based assays, while specific for T cells recognizing an antigen/MHC complex of interest, are indirect assays and difficult to use to accurately define the number of T cells of interest. Thus, a major advance that ^{125}I MHC/Ig complexes provide is the ability to directly identify antigen-specific T cells in development of an immune response.

We studied the ability to use ^{125}I MHC/Ig complexes to analyze T cell

repertoire *in vivo* by staining spleen cells from animals immunized with a model tumor vaccine, CT26-GM. CT26-GM is a murine colon carcinoma line that has been transfected with a gene encoding GM-CSF. When animals are immunized with CT26-GM they become resistant to growth of the parental CT26 tumor. This resistance is due to T cells that develop in immunized animals and that recognize a peptide antigen, AH1, presented in the context of H-2L^d molecules. AH1 peptides are derived from an endogenous murine retrovirus whose sequence we have previously defined and which is known to bind effectively to H-2L^d molecule and to sensitize T cells for development of an anti-CT26 immune response.

^{pcp}MHC/Ig complexes efficiently detect antigen-specific T cells in animals immunized with CT26-GM. Fourteen days after a single immunization with CT26-GM cells, ~ 3% of all CD8⁺ T cells are reactive with AH1L^d/Ig complexes. Control ^{pcp}MHC/Ig complexes, ^{β-gal}L^d/Ig and ^{MCMV}L^d/Ig, are reactive with approximately 10-fold fewer cells, or 0.3% of all CD8⁺ T cells. In a naive, nonimmunized animal, AH1L^d/Ig complexes are only reactive with ~ 0.3% of all CD8⁺ T cells. Therefore a single immunization leads to an approximately 10-fold increase in the total number of antigen-reactive T cells which could be detected using ^{pcp}MHC/Ig. Thus, ^{pcp}MHC/Ig can be used to monitor T cell homeostasis following immunization, transplantation, or during treatment of antigen-specific autoimmune diseases.

These results demonstrate that these reagents can be used with flow cytometry to specifically stain for T cells from lymphoid organs, peripheral blood, or other infiltrated tissues, as a means of directly and quantitatively assessing the number of antigen-specific T cells. Use of these reagents in combination with markers for activation provides a quantitative analysis of the activation state of these T cells. In addition, by using these reagents with a cell sorter, purified populations of antigen-specific T cells can be sorted. The purified populations can then be manipulated *in vitro*.

EXAMPLE 6

This example demonstrates that peptide loaded $Q^{19}L^d/Ig$ or $^{SIY}K^b/Ig$ specifically blocks lysis of target cells by 2C cytotoxic lymphocytes (CTL).

5 Using $Q^{19}L^d/Ig$ as an inhibitor complex, target cell lysis was markedly inhibited, from 82% down to 2% (Figures 9A and 9B). Half maximal inhibition occurred at approximately 10 nM of $Q^{19}L^d/Ig$. Control $^{PCP}MHC/Ig$ complexes, $^{MCMV}L^d/Ig$, had no impact on lysis of target cells. The anticolonotypic monoclonal antibody, 1B2, was also used as a positive control antibody which inhibited CTL mediated lysis.

10 $^{SIY}K^b/Ig$ also dramatically inhibited 2C CTL mediated lysis of target cells. In this case, maximal inhibition lowered specific lysis down from 85% to 35%. Inhibition of CTL mediated lysis is of particular importance, since CTL lytic activity can detect a very low concentration of antigen/MHC complexes on target cells. Thus, CTL-mediated lysis is considered very
15 difficult to inhibit.

In addition to lysis of target cells, a variety of other physiologic responses, including T cell proliferation and lymphokine secretion, are associated with activation of $CD8^+$ T cell activation. Peptide-loaded divalent MHC, $^{SIY}K^b/IgG$, also inhibited T cell proliferation of 2C CTL stimulated by
20 allogeneic splenic cells. Maximal inhibition was seen at about 20 nM $^{SIY}K^b/IgG$; the stimulation index of untreated cells decreased from 100% to 10% for cells treated with 20 nM $^{SIY}K^b/IgG$. Half maximal inhibition was seen at approximately 1 nM $^{SIY}K^b/IgG$.

25 The ability to selectively block antigen-specific immune responses provides a means by which this reagent can be used, for example, to turn off pathogenic responses in autoimmune disease or responses that lead to rejection after organ transplantation.

30 Modification of these reagents can also be made to allow the reagents to specifically kill antigen-specific T cells as a more definitive way to suppress these antigen immune responses. For example, the reagent can be conjugated with a toxin molecule, such as ricin or the α chain of *Psuedomonas*

endotoxin. According to such an embodiment, the toxin-conjugated reagent is either injected *in vivo* or applied *in vitro*. When the reagent binds to T cells specific for the peptide/MHC complex, the reagent is internalized. The toxin molecule then selectively kills the pathogenic T cell of interest.

5 **EXAMPLE 7**

This example demonstrates activation of antigen-specific cells.

Because ^{Pc}P/MHC/Ig complexes crosslink cell surface T cell receptors, ^{Pc}P/MHC/Ig complexes can also be used to stimulate T cells. Immobilization of ^{Pc}P/MHC/Ig can stimulate antigen-specific T cells. Thus, these reagents can be
10 used to selectively activate antigen-specific T cells either *in vitro* or *in vivo*.

The reagents can be used to activate T cells *in vitro*, followed by adoptive transfer into a patient. Alternatively, the reagents can be injected into a patient as a means of activating tumor antigen-specific T cells *in vivo*. Thus, the reagents of the invention represent a potent therapeutic antigen-
15 specific vaccine for cancer.

These reagents can also be used as immunotherapeutics for infectious diseases, such as fungal disease, microbacterial diseases, viral diseases such as HIV, or intracellular parasites to activate T cell specific for antigens expressed for these pathogens.

20 The reagents of the invention thus represent a means of activating a variety of antigen-specific T cell responses.

EXAMPLE 8

This example demonstrates the covalent linkage of peptides for expression as part of a class I MHC/Ig chimera.

25 Another modification that provides for more stable production of these multivalent MHC/Ig reagents loaded with peptides of interest is the covalent linkage of the peptide via a linker to the MHC molecule. For MHC class I molecules, this is most easily accomplished by linking the peptide to the amino terminus of β 2 microglobulin. For MHC class II molecules, the

peptide can be tethered via a linker to the amino terminus of the β chain of MHC class II, as previously described. Using these tethered peptide MHC constructs, the peptide of interest is closely linked to the MHC molecule via the tether. This linkage favors the specific binding of that peptide over other potentially interfering peptides in the serum or medium.

To tether a peptide to the amino terminus of β 2M, we have started by tethering a peptide derived from tax 11-19 to β 2M (Figure 19). The amino terminus of β 2M was modified by genetically linking DNA encoding the linker with a convenient cloning site for insertion of different peptide into the amino terminus of β 2M. A multiple round PCR-based mutagenesis was used to generate the peptide tethered to β 2M. Oligonucleotides 1 and 2 were used to generate a small PCR fragment containing the human β 2M leader fused to the tax 11-19 peptide. This PCR product was then used as a template for another PCR reaction using oligos 1 and 3. The PCR fragment generated from this second reaction covers the entire region, approximately 110 base pairs, between the Xho I and Bsp EI cloning sites.

This fragment was digested and cloned into the modified human β 2M construct. Using this construct one can easily cassette in any peptide of interest to the amino terminus of β 2M. For expression, the modified human β 2M cDNA was cloned into pcDNA3.1/Zeo- and cotransfected with the chimeric HLA-A2/Ig construct into J558L cells. Resultant cells can be selected with both G418 and Zeocin to ensure expression of both the chimeric HLA-A2/Ig and β 2M polypeptides.

Peptide-tethered tax- β 2M complexed with HLA-A2/Ig was specific in its interaction with HLA-A2 restricted CTL clones analyzed by flow cytometry. Thus, Tax tethered-A2/Ig was specifically reactive with an HLA-A2-restricted, HTLV-1 Tax 11-19-specific CTL clone, A6 (22). Mean channel fluorescence (MCF) of A6 cells stained with peptide tethered tax- β 2M HLA-A2/Ig complexes was approximately 4000, MCF of A6 cells stained using no chimeric protein was only 10 and almost identical to staining controls with the control Gag-A2/Ig complex, unloaded HLA-A2/Ig. The

level of staining with tax-b2M was similar to that obtained with an anti-V β 13.1 specific mAb, indicating that all the relevant sites were saturated using tax- β 2M HLA-A2/Ig. Thus peptide tethering to β 2M is an efficient way to present peptides in the context of MHC/Ig molecules.

5 *Direct Visualization of Antigen-Specific T Cells in HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis*

10 The following examples demonstrate the use of reagents of the invention to visualize directly antigen-specific T cells in patients with HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and HIV infections.

15 HAM/TSP is caused by a human retrovirus, human T lymphotropic virus type I (HTLV-1) (2, 3). The clinical symptoms of HAM/TSP are characterized by upper motor neuron signs and mild sensory and sphincter dysfunction (3). Histopathologically, one finds thoracic spinal cord atrophy involving perivascular demyelination and axonal degeneration (4, 5).

20 The pathophysiology of HAM/TSP is still not completely understood (6). Serum reactivity to HTLV-1 was first associated with HAM/TSP by Osame and coworkers in 1986 (3). The immunologic hallmark of patients with HAM/TSP is the spontaneous proliferation of peripheral-blood lymphocytes (PBL) *in vitro* (7-9).

25 It has been previously demonstrated that circulating CD8⁺ cytotoxic T lymphocytes (CTL) in patients with HAM/TSP react against HTLV-1 protein products (10) and an immunodominant HLA-A2-restricted epitope (HTLV-1 Tax11-19) has been well characterized (11). HTLV-1 Tax11-19-specific CTL precursor frequency was estimated by limiting dilution analysis in the range of 1/75 to 1/320 CD8⁺ lymphocytes (12) in peripheral blood. Tax-specific CTL were also found in cerebrospinal fluid (CSF) (12, 13) and HTLV-1 specific clones could be generated *in vitro* from a spinal cord lesion that was obtained from a HAM/TSP patient (14).

30 Immunohistochemical analysis of affected spinal cord lesions in the early stage of the disease from patients with HAM/TSP reveals the presence of

infiltrating CD4⁺ and CD8⁺ lymphocytes, in which the CD8⁺ population predominates with duration of the disease (15). Moreover an increase in activated lymphocytes has been shown in PBL and CSF (16, 17). Recently, we have been able to demonstrate that peripheral CD8⁺ T lymphocytes produce IL-2, IFN- γ and TNF- α in HAM/TSP patients (18). Collectively, this evidence has supported the view that virus-specific CD8⁺ T lymphocytes may play a critical role in the immunopathogenesis of HAM/TSP.

One major limitation in these studies has been the inability to identify HTLV-1 specific CD8⁺ T cells directly from biological samples not only to quantitate the actual number of antigen-specific T cells *in vivo* but also to further characterize the antigen-specific population of T lymphocytes without *in vitro* amplification. To overcome these problems, we have developed divalent MHC class I constructs using immunoglobulin as a scaffold (20) and used these to elucidate the role of antigen-specific CD8⁺ T cells in different human immunologic diseases, such as HAM/TSP.

The examples below demonstrate our analysis of HTLV-1 Tax11-19- and p17 Gag77-85-specific, HLA-A2⁺-restricted CD8⁺ T lymphocytes directly from peripheral blood and cerebrospinal fluid using peptide-loaded divalent HLA-A2/Ig chimeras.

EXAMPLE 9

This example demonstrates materials and methods used to analyze HLA-A2⁺-restricted CD8⁺ T lymphocytes specific for HTLV-1 Tax11-19 or p17 Gag77-85 directly from peripheral blood and cerebrospinal fluid using peptide-loaded divalent HLA-A2/Ig chimeras.

Study subjects and specimens. Clinical characteristics of patients with HAM/TSP and asymptomatic HTLV-1 infected individuals as well as HLA type were previously described (12) and are summarized in Table 1. HTLV-1 infection was confirmed by Western Blot in serum of HAM/TSP patients and asymptomatic carriers. Diagnosis of HAM/TSP was made according to WHO criteria, by reference to neurological symptoms and serological testing

of HTLV-1.

PBL were prepared by Ficoll-Hypaque centrifugation and stored in liquid nitrogen until use. Histocompatibility typing was performed on PBL or EBV-transformed lymphoblastoid cell lines by the Tissue Typing Laboratory at the NIH Clinical Center. Some of the healthy HLA-A2 uninfected controls were tested for HLA-A2 expression by FACS analysis using a panel of HLA-A2-specific monoclonal antibodies: MA2.1, BB7.2 and PA2.1 (American Type Culture Collection, Rockville, MD).

Peptides. Peptides HTLV-1 Tax11-19 (LLFGYPVYV) (SEQ ID NO:1), influenza virus A M158-66 (GILGFVFTL) (SEQ ID NO:2), and p17 Gag77-85 (SLYNTVATL) (SEQ ID NO:3) were synthesized and HPLC purified at the Colorado State University Macromolecular Resources. Identity of the peptides was confirmed by mass spectrometry measurement.

Cloning the construct and protein expression. Using oligonucleotide-directed PCR, we introduced a 5' Mlu I and a 3' Not I site (sites underlined) into the extracellular domain (α 1- α 3) of HLA-A2 cDNA using the following primer pair:

5'-GATACGCGTTGGGCTCTCACTCCATGAG (SEQ ID NO:4)
and 5'-CAGTCGATGCGGCCGCCATCTCAGGGTGAGGGGCT (SEQ ID NO:5). After PCR amplification, the α 1- α 3 region of HLA-A2 was sequenced and directionally cloned in exchange for H-2K^b into the previously described pX/Ig vector (20).

The construct was cotransfected with DNA encoding the chimeric HLA-A2/Ig protein and the human β_2 -microglobulin by electroporation into J558L cells. Transfectants were screened for secretion of the chimeric protein by ELISA as previously described (20). ELISA plates were coated with BB7.2, an antibody specific for peptide-loaded conformations of HLA-A2, or with a goat anti-mouse IgG-Fc antibody (10 μ g/ml) (Cappel, Durham, NC). High secretors were picked and grown in Hybridoma-SFM (Gibco-BRL, Gaithersburg, MD). HLA-A2/Ig secretion from the transfected J558L was confirmed by SDS-PAGE electrophoresis.

The chimeric protein was harvested from cell supernatant and was concentrated with Centriflo®-membrane cones (Amicon, Beverly, MA). HLA-A2/Ig was loaded with 660 fold-molar excess of peptide for 10-14 days prior to FACS analysis. Three μg of protein was used for staining of 1×10^6 cells.

Flow Cytometric Analysis. Murine monoclonal anti-human CD8-FITC (Sigma, St. Louis, MO), anti CD-4 PE (Becton Dickinson, San Jose, CA), anti-CD8-Tri (Caltag Laboratories, Burlingame, CA), and anti-HLA-DR (Pharmingen, San Diego, CA) were used to detect cell surface molecules of lymphocytes. PK1.36-FITC was used as an isotype-matched control for anti-HLA DR-FITC. HLA-A2/Ig bound onto the cell surface was detected using goat-anti-mouse IgG1-PE or a goat-anti-mouse IgG1-Tri (Caltag). Monoclonal anti-TNF- α -FITC, anti-IFN- γ -FITC, or isotype-matched IgG1-FITC antibodies (Pharmingen) were used for intracellular cytokine staining. PBL (1×10^6) were incubated with peptide-loaded HLA-A2/Ig on ice, followed by PE or Tri-conjugated goat-anti mouse IgG1-PE.

Intracellular staining was performed according to the manufacturer's instructions with slight modifications (21) after a 4 hour incubation at 37°C , followed by a 10 h incubation in the presence of $10 \mu\text{g/ml}$ brefeldin A (Sigma). Three-color fluorimetric analysis was carried out on a FACScan (Becton Dickinson). Lymphocytes were gated on forward and side scatter. 1×10^5 (2×10^5 for three-color analysis) gated events were analyzed using CELLQuest software (Becton Dickinson). PBL from all HLA-A2 positive patients were stained at least twice with similar results.

EXAMPLE 10

This example demonstrates that peptide-loaded HLA A2/Ig binds specifically HLA-A2 restricted CTL.

We have engineered a divalent HLA-A2/Ig molecule, using immunoglobulin as a scaffold, that can be loaded with various peptides. Peptide-loaded HLA-A2/Ig was specific in its interaction with HLA-A2

restricted CTL clones analyzed by flow cytometry. Thus, Tax-A2/Ig was specifically reactive with an HLA-A2-restricted, HTLV-1 Tax11-19-specific CTL clone, A6 (22). Mean channel fluorescence (MCF) of A6 cells stained with HTLV-1 Tax11-19 loaded HLA-A2/Ig (Tax-A2/Ig) was 426, while
5 MCF of A6 cells stained with the control Gag-A2/Ig complex was only 13 and almost identical to staining controls using no chimeric protein, unloaded HLA-A2/Ig, or M1-loaded HLA-A2/Ig (Figure 11A). In complementary experiments, Gag-A2/Ig specifically stained the HIV p17 Gag 77-85-specific clone SL09 (23) (Figure 11B), while staining with Tax-A2/Ig was virtually
10 identical to unloaded HLA-A2/Ig, M1-loaded HLA-A2/Ig, or no HLA-A2/Ig.

EXAMPLE 11

This example demonstrates visualization of antigen-specific T lymphocytes in peripheral blood of patients with HAM/TSP and HIV infections.

15 HTLV-1 Tax11-19-specific CTL activity has been demonstrated directly in PBL from HAM/TSP patients (10). In HLA-A2⁺ patients, HTLV-1-specific reactivity is predominately directed against the HTLV-1 Tax11-19 epitope (11). Previous estimates of precursor frequencies of HTLV-1 Tax11-19-specific CTL using limiting dilution analysis (LDA) gave
20 values in the range of 1/75 to 1/320 CD8⁺ T cells.

To quantitate directly the HTLV-1 Tax11-19-specific CD8⁺ population in peripheral blood of HAM/TSP patients, we performed two dimensional flow cytometric analysis of cells using Tax-A2/Ig and anti-CD8⁺ (Figure 12). PBL from patients with HAM/TSP showed a significant high number of HTLV-1
25 Tax11-19 specific CD8⁺ T lymphocytes. In one patient the frequency of Tax11-19-specific cells was as high as 13.8% (Figures 12 and 13 and Table 1). The percentage of Tax-A2/Ig specific cells ranged between 0.64 and 13.87% of CD8⁺ T cells, with values >2% for 4 of the 6 HAM/TSP patients (Figure 12). CD8⁺ T cells specific for p17 Gag77-85 could also be detected
30 (Figure 12, right panels).

In one of the six HLA-A2 positive HAM/TSP patients, no Tax-A2/Ig-reactive cells were detected. However, this same patient's PBL also did not lyse Tax protein-expressing, autologous, EBV transformed B cells (unpublished data). Moreover, we were able to detect Tax-specific T cells with Tax-A2/Ig in every patient sample that lysed Tax-expressing, HLA-A2⁺ target cells (Table 1).

EXAMPLE 12

This example demonstrates analysis of peripheral blood from two HLA-A2⁺ HTLV-1 carriers.

We also analyzed peripheral blood from two HLA-A2⁺ HTLV-1 carriers who did not have HAM/TSP (Figure 13 and Table 1). One of the two HTLV-1 carriers had no detectable HTLV-1 Tax11-19-specific T cells, while the other contained a low but reproducibly detectable number of CD8⁺ T cells that stained with Tax-A2/Ig (0.33%).

To confirm the specificity of staining of peripheral blood samples with these reagents and their relationship to the disease, we analyzed PBL from HAM/TSP patients who do not carry the HLA-A2 allele (Table 1 and Figure 13), uninfected healthy HLA-A2⁺ donors (Table 1 and Figure 13), and an HIV-1 infected HLA-A2⁺ individual (Figure 12). Less than 0.1% of CD8⁺ T cells from all of these samples stained positively with the Tax-A2/Ig. However, a significant number (3.03%) of the CD8⁺ T lymphocytes from the HIV-infected individual was stained using Gag-A2/Ig.

Collectively, these results demonstrate that the presence of high numbers of Tax-A2/Ig staining CD8⁺ cells is specific for HTLV-1⁺ HLA-A2⁺ individuals with HAM/TSP.

EXAMPLE 13

This example demonstrates visualization of antigen-specific T lymphocytes in cerebrospinal fluid of patients with HAM/TSP.

To corroborate the significance of HTLV-I-specific CTLs for the

pathogenesis of this demyelinating disease, we analyzed lymphocytes from cerebrospinal fluid of a patients with HAM/TSP (H1) and compared them with the T cells in his peripheral blood from the same day. Tax-A2/Ig-specific CD8⁺ cells constituted 23.7% of the CD8⁺ cell population in cerebrospinal fluid were. In contrast, we detected 9.4% Tax-A2/Ig specific CD8⁺T cells in the peripheral blood (Figure 14A). Interestingly, we found the same number of CD4⁺ T cells in peripheral blood and cerebrospinal fluid (44% in the PBL and 48% in the CSF), but also an increased number in total CD8⁺ T cells in the CSF (42%) compared to the peripheral blood (29%) (Figure 14B).

EXAMPLE 14

This example demonstrates that HTLV-1 Tax11-19-specific T cells in patients with HAM/TSP are activated.

The ability to stain HTLV-1 Tax11-19-specific T lymphocytes directly provides the opportunity to analyze their state of activation. If HTLV-1-specific CD8⁺ T cells indeed mediate the immunopathogenesis of HAM/TSP, a significant proportion of them might be expected to be activated. Since activated human T cells express elevated levels of MHC class II molecules, we further characterized the HTLV-1 Tax11-19-specific T cells from HAM/TSP patients for HLA-DR expression by multicolor flow cytometry.

Tax-A2/Ig reactive, CD8⁺ T cells from the 3 HAM/TSP patients had significantly increased expression of DR (Figure 15). In 2 of 3 patients, the level of DR expression was virtually identical to that seen in control PHA-stimulated PBL (compare Figures 15A and 15B, Tax-A2/Ig reactive-specific CD8⁺ T cells from patients, to Figure 15E, activated human PBL). Similar results were obtained for IL-2 receptor expression (data not shown).

Of particular interest, one patient, H6, demonstrated a significantly lower population of DR⁺, Tax-specific CD8⁺ cells (Figure 15C). This individual, the spouse of a HAM/TSP patient, was originally thought to be an asymptomatic carrier, since no neurologic symptoms were reported.

However, on a physical examination a hyperreflexia in the lower extremities and extensor plantar responses were observed, indicative of corticospinal tract lesion(s).

EXAMPLE 15

5 This example demonstrates expression of cytokines by Tax-specific CD8⁺ cells from HAM/TSP patients.

 Inflammatory cytokines are considered critical mediators of CNS immunopathology in many autoimmune models. Elevated levels of several proinflammatory cytokines have been detected in the serum or cerebrospinal
10 fluid of HAM/TSP patients (16, 17). Therefore it is of particular interest to analyze Tax-specific CD8⁺ cells from HAM/TSP patients for the presence of relevant intracellular cytokines.

 Recently, we have found that bulk CD8⁺ T cells from HAM/TSP patients demonstrated significant expression of cytokines, such as TNF- α , IFN- γ , and
15 IL-2 but not IL-4 (18). Tax-specific CD8⁺ T cells from patients H1 (Figure 16), H5 (data not shown), and H6 (Figure 16) demonstrated distinct patterns of intracellular TNF- α and IFN- γ expression. While the HAM/TSP patients H1 and H5 displayed a high proportion of Tax-A2/Ig-specific, CD8⁺ cells with intracellular TNF-a and IFN-g (roughly 30%), patient H6 had a very low
20 proportion of Tax-specific CD8⁺ cells expressing intercellular TNF-a and IFN-g (2%).

 These results, together with HLA-DR analysis, demonstrate a disassociation between expansion of antigen-specific T cells and their activation state. They further demonstrate how the ability to use
25 multiparameter flow cytometry to analyze the activation state of antigen-specific T cell populations can provide further insight into the relationship between state of T cell activation and disease pathogenesis.

EXAMPLE 16

 This example demonstrates that Tax-Specific CD8⁺ T Cells Persist in

Peripheral Blood from HAM/TSP Patients.

An important aspect in understanding the pathogenesis of autoimmune diseases, such as HAM/TSP is to understand the dynamics of antigen-specific T cell responses. We therefore compared the numbers of Tax-specific, CD8⁺ T cells from a HAM/TSP patient (H1) who had serial PBL drawn between 1989 and 1998. This patient was initially diagnosed in 1979.

Interestingly, even 10 years after diagnosis, this patient had about 10% Tax-specific CD8⁺ T cells. This percentage did not change significantly over the next eight years (Figure 17).

Thus, in this patient, pathogenic Tax-specific CD8⁺ T cells are sustained at high levels even after 19 years of active disease.

TABLE 1

Donor	Gender/Age	clinical status ¹	duration of disease (years)	HLA-A	CTL activity ²	CD8 ⁺	Tax ⁺ CD8 ⁺ ³	M1 ⁺ CD8 ⁺ ⁴	% Tax-spec. CD8 ⁺ ⁵
H1 ⁶	M / 52-60	HAM/TSP	15-23	A 2, 28	+	21.46	2.52	0.04	7.24 - 13.87
H2	F / 47	HAM/TSP	16	A 2, 33	+	21.14	0.18	0.04	0.64
H3	F / 48	HAM/TSP	2	A 2, 3	-	16.66	0.02	0.02	0
H4	M / 58	HAM/TSP	14	A 2, 66	+	24.52	0.82	0.04	3.08
H5	F / 51	HAM/TSP	16	A 2, 3	+	14.85	0.49	0.04	2.94
H6	F / 77	HAM/TSP	4	A 1, 2	+	45.38	1.62	0.10	3.23
C1	F / 54	carrier	-	A 2, 11	+	21.41	0.10	0.03	0.33
C2	F / 52	carrier	-	A 2, 24	-	22.33	0.01	0.01	0
1	n.d.	healthy	-	A 2	-	41.48	0.03	0.02	0.02
2	n.d.	healthy	-	A 2	n.d.	22.31	0.03	0.13	0
3	n.d.	healthy	-	A 2	n.d.	14.47	0.09	0.08	0.08
4	n.d.	healthy	-	A 2	n.d.	27.98	0.01	0.03	0
5	M / 31	healthy	-	A 2	-	25.41	0.02	0.09	0
H7	M / 78	HAM/TSP	10	A 1, 3	+	22.34	0.06	0.3	0.07
H8	F / 37	HAM/TSP	8	A 11, 24	+	28.29	0.03	0.02	0.04
H9	F / 49	HAM/TSP	10	A 23, 33	+	27.77	0.02	0.02	0

Table 1:

¹ Carriers were identified among HTLV-1 infected spouses of HAM/TSP patients

² CTL activity was previously determined from peripheral blood using Epstein Barr virus immortalized B cells infected with vaccinia expressing the Tax gene as targets (10, 12) and unpublished data.

³ Percent of cells that stained positive for CD8⁺ expression and HTLV-1 Tax-A2/Ig

⁴ Percent of cells that stained positive for CD8⁺ expression and HLA-A2/Ig loaded with an irrelevant M1 peptide.

⁵ Percent of CD8⁺ cells that stained positive for Tax-A2/Ig (Background staining using M1-HLA-A2/Ig was subtracted out). All HTLV-1 infected individuals were stained at least twice.

⁶ Samples between 1989 to 1998 were tested see Figure 18.

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We claim:

1. A composition comprising a chimeric protein which comprises an MHC molecule and an immunoglobulin chain, wherein the chimeric protein associates to form molecular complexes comprising at least two chimeric proteins per complex, wherein an antigenic peptide is bound to each MHC molecule, wherein each MHC molecule within the molecular complex is bound to an identical antigenic peptide.
2. The composition of claim 1 wherein each MHC molecule in the composition is bound to an identical antigenic peptide.
3. The composition of claim 1 wherein the antigenic peptide is passively bound.
4. The composition of claim 1 wherein the antigenic peptide is actively bound.
5. The composition of claim 1 wherein the antigenic peptide is covalently bound to the MHC molecule.
6. The composition of claim 1 wherein the antigenic peptide is bound to the amino-terminus of $\beta 2$ microglobulin.
7. The composition of claim 1 wherein the antigenic peptide is bound to the amino-terminus of an MHC class II β chain.
8. The composition of claim 7 wherein the antigenic peptide is bound to the amino-terminus of the MHC class II β chain by means of a peptide tether.
9. The composition of claim 1 wherein the molecular complex comprises two of said chimeric proteins.
10. The composition of claim 1 wherein the molecular complex is conjugated to a toxin.
11. The composition of claim 1 wherein the MHC molecule is an MHC class I molecule.
12. The composition of claim 11 wherein the MHC class I molecule is an HLA class I molecule.
13. The composition of claim 12 wherein the MHC molecule is an

HLA-A2 molecule.

14. The composition of claim 1 wherein the antigenic peptide is selected from the group consisting of an HTLV-Tax11-19 peptide, a p17 Gag77-85 peptide, and an influenza virus A M158-66 peptide.

5 15. A vector encoding a chimeric protein comprising an immunoglobulin heavy chain and an MHC molecule, wherein the immunoglobulin is not IgG-1, wherein the immunoglobulin heavy chain is C-terminal to the MHC molecule.

10 16. The vector of claim 15 wherein the MHC molecule is an MHC class I molecule.

17. The vector of claim 16 wherein the MHC class I molecule is an HLA class I molecule.

18. The vector of claim 17 wherein the HLA class I molecule is an HLA-A2 molecule.

15 19. A composition comprising a chimeric protein which comprises an MHC molecule and an Ig chain, wherein the chimeric protein associates to form molecular complexes comprising at least two chimeric proteins per complex, wherein the immunoglobulin chain is not an IgG heavy chain.

20 20. The composition of claim 19 wherein the chimeric protein is conjugated to a toxin.

25 21. A composition comprising a cell in which a chimeric protein is bound to the surface of the cell, wherein the chimeric protein comprises an MHC molecule and an immunoglobulin chain; wherein the chimeric protein associates to form molecular complexes comprising at least two chimeric proteins per complex, wherein an antigenic peptide is bound to each MHC molecule, wherein each MHC molecule within the molecular complex is bound to an identical antigenic peptide.

22. The composition of claim 21 wherein the cell is a dendritic cell.

30 23. The composition of claim 21 wherein a population of said chimeric proteins is bound to the cell, wherein each MHC molecule in the population of said chimeric proteins is bound to an identical antigenic peptide.

24. A method for treating a patient suffering from an allergy,
comprising:

5 administering to the patient at a dose sufficient to suppress a T cell
response associated with an allergy of the patient a chimeric protein which
comprises an MHC molecule and an immunoglobulin chain; wherein the
chimeric protein associates to form molecular complexes comprising at least
two chimeric proteins per complex, wherein an antigenic peptide is bound to
each MHC molecule, wherein each MHC molecule within the molecular
complex is bound to an identical antigenic peptide, and wherein the antigenic
10 peptide is an antigen to which the patient has an allergic response.

25. A method for treating a patient who has received or will receive an
organ transplant, comprising:

15 administering to the patient at a dose sufficient to suppress an
immune response to the transplanted organ a chimeric protein which
comprises an MHC molecule and an immunoglobulin chain; wherein the
chimeric protein associates to form molecular complexes comprising at least
two chimeric proteins per complex, wherein an antigenic peptide is bound to
each MHC molecule, wherein each MHC molecule within the molecular
complex is bound to an identical antigenic peptide, and wherein the antigenic
20 peptide is an alloantigen.

26. A method for treating a patient suffering from an autoimmune
disease, comprising:

25 administering to the patient at a dose sufficient to suppress an
immune response associated with the autoimmune disease a chimeric protein
which comprises an MHC molecule and an immunoglobulin chain; wherein
the chimeric protein associates to form molecular complexes comprising at
least two chimeric proteins per complex, wherein an antigenic peptide is
bound to each MHC molecule, wherein each MHC molecule within the
molecular complex is bound to an identical antigenic peptide, and wherein the
30 antigenic peptide is one to which the patient expresses an autoimmune
response.

27. The method of claim 26 wherein the autoimmune disease is HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP).

28. The method of claim 26 wherein the MHC molecule is an MHC class I molecule.

5 29. The method of claim 28 wherein the MHC class I molecule is an HLA class I molecule.

30. The method of claim 29 wherein the HLA class I molecule is an HLA-A2 molecule.

10 31. The method of claim 27 wherein the antigenic peptide is HTLV-1 Tax11-19.

32. A method for treating a patient having a tumor, comprising:
administering to the patient at a dose sufficient to induce or
enhance an immune response to the tumor a chimeric protein which comprises
an MHC molecule and an immunoglobulin chain; wherein the chimeric protein
15 associates to form molecular complexes comprising at least two chimeric
proteins per complex, wherein an antigenic peptide is bound to each MHC
molecule, wherein each MHC molecule within the molecular complex is
bound to an identical antigenic peptide, and wherein the antigenic peptide is a
tumor-associated peptide.

20 33. A method for treating a patient having an infection caused by an infectious agent, comprising:

administering to the patient at a dose sufficient to induce or
enhance an immune response to the infection a chimeric protein which
comprises an MHC molecule and an immunoglobulin chain; wherein the
25 chimeric protein associates to form molecular complexes comprising at least
two chimeric proteins per complex, wherein an antigenic peptide is bound to
each MHC molecule, wherein each MHC molecule within the molecular
complex is bound to an identical antigenic peptide, and wherein the antigenic
peptide is a peptide of an infectious agent.

30 34. The method of claim 33 wherein the infection is an HIV infection.

35. The method of claim 34 wherein the antigenic peptide is p17

Gag77-85.

36. The method of claim 33 wherein the MHC molecule is an MHC class I molecule.

37. The method of claim 36 wherein the MHC class I molecule is an HLA class I molecule.

38. The method of claim 37 wherein the HLA class I molecule is an HLA-2 molecule.

39. The method of claim 33 wherein the infection is an influenza infection.

40. The method of claim 39 wherein the antigenic peptide is an influenza virus A M158-66 peptide.

41. A method of labeling antigen-specific T cells, comprising:
contacting a sample which comprises antigen-specific T cells with a chimeric protein; wherein the chimeric protein comprises an MHC molecule and an immunoglobulin chain, wherein the chimeric protein associates to form molecular complexes comprising at least two chimeric proteins per complex, and wherein each MHC molecule within the molecular complex is bound to an identical antigenic peptide, whereby the antigenic peptide specifically binds to the antigen-specific T cells, thereby labeling the cells with the chimeric protein.

42. The method of claim 41 further comprising the step of:
separating the antigen-specific T cells which are bound to the antigenic peptides from cells which are not bound.

43. The method of claim 42 wherein the step of separating is performed using flow cytometry.

44. The method of claim 41 further comprising the step of:
counting the number of antigen-specific T cells which are bound to the antigenic peptides.

45. The method of claim 41 wherein said step of contacting is performed *in vitro*.

46. The method of claim 41 wherein said step of contacting is

performed *in vivo*.

47. The method of claim 41 wherein the MHC molecule is an MHC class I molecule.

5 48. The method of claim 47 wherein the MHC molecule is an HLA class I molecule.

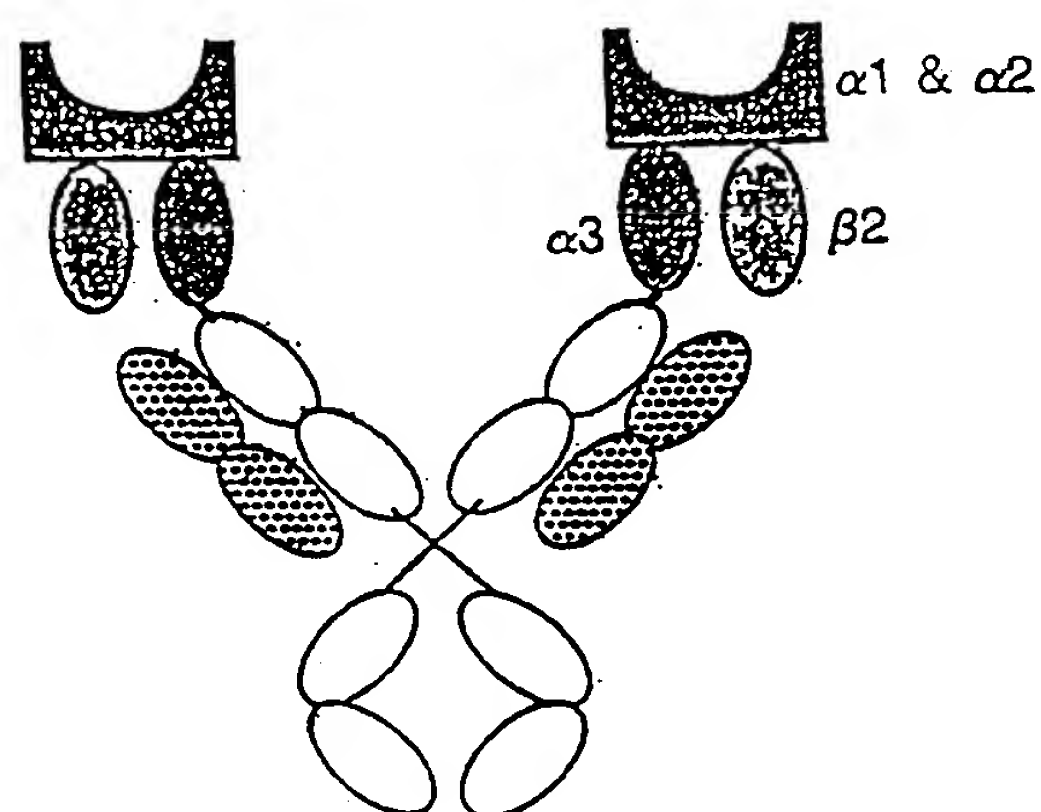
49. The method of claim 48 wherein the HLA class I molecule is an HLA-A2 molecule.

10 50. The method of claim 41 wherein the antigenic peptide is selected from the group consisting of an HTLV-1 Tax11-19 peptide, a p17 Gag77-85 peptide, and an influenza virus A M158-66 peptide.

51. The method of claim 41 further comprising the step of:
detecting a marker for activation of the antigen-specific T cells.

52. The method of claim 51 wherein the marker for activation of antigen-specific T cells is a secreted lymphokine.

FIG. 1A



CAGGTCCAACCTGCACGCGTTGGGCTCT $\alpha 1-\alpha 3$ AGATGGGCGGCCGCACTGGAGGTGAGCCAGGTC
Q V Q L H A L G S R W A A A L E V S Q V
1 2 3 4 LINK 25 26 LINK 1 2

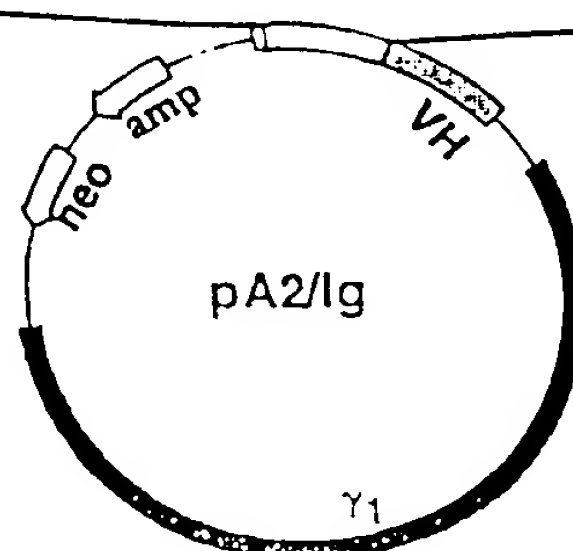


FIG. 1B

FIG. 2

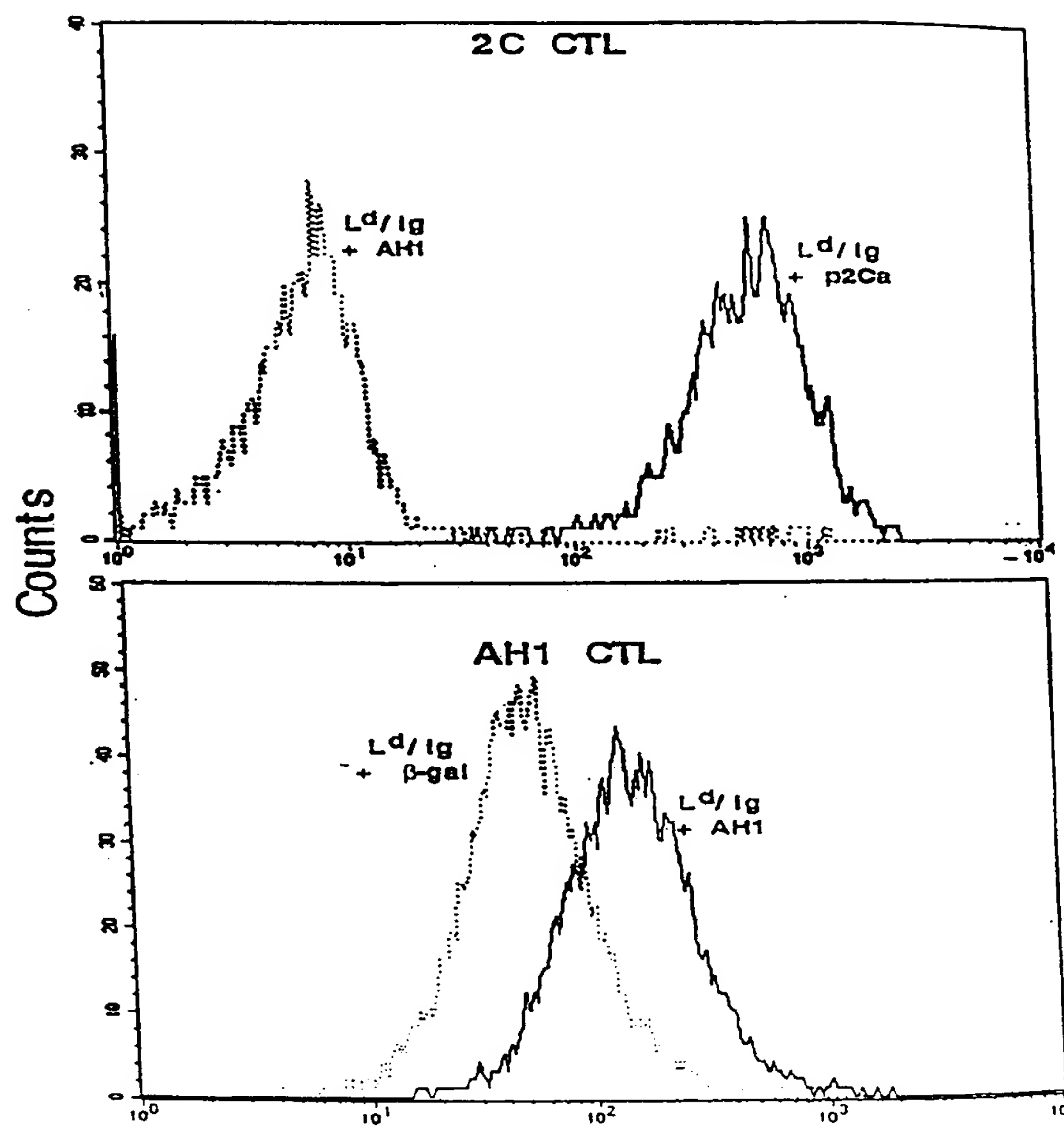


FIG. 3

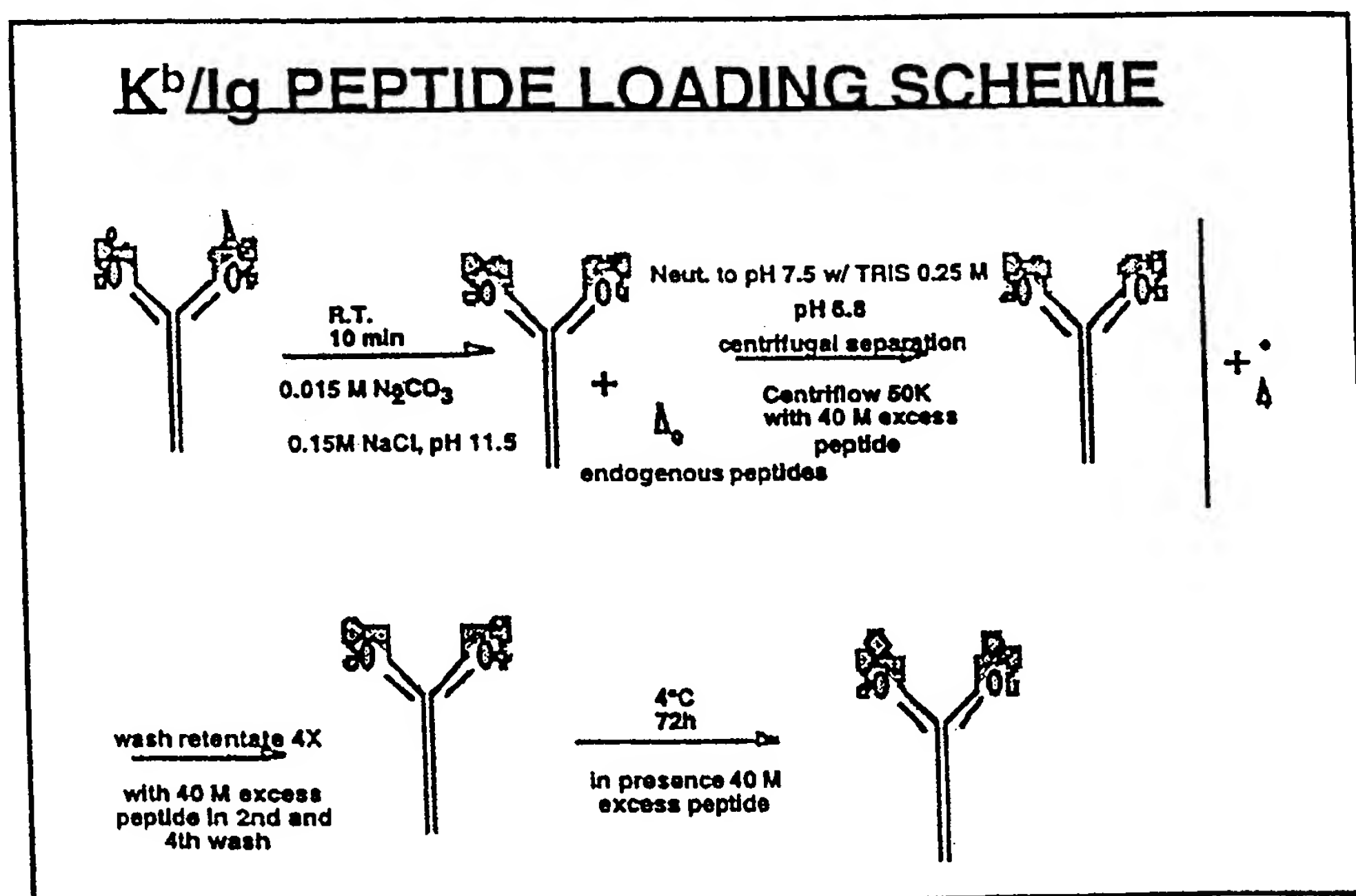


FIG. 4

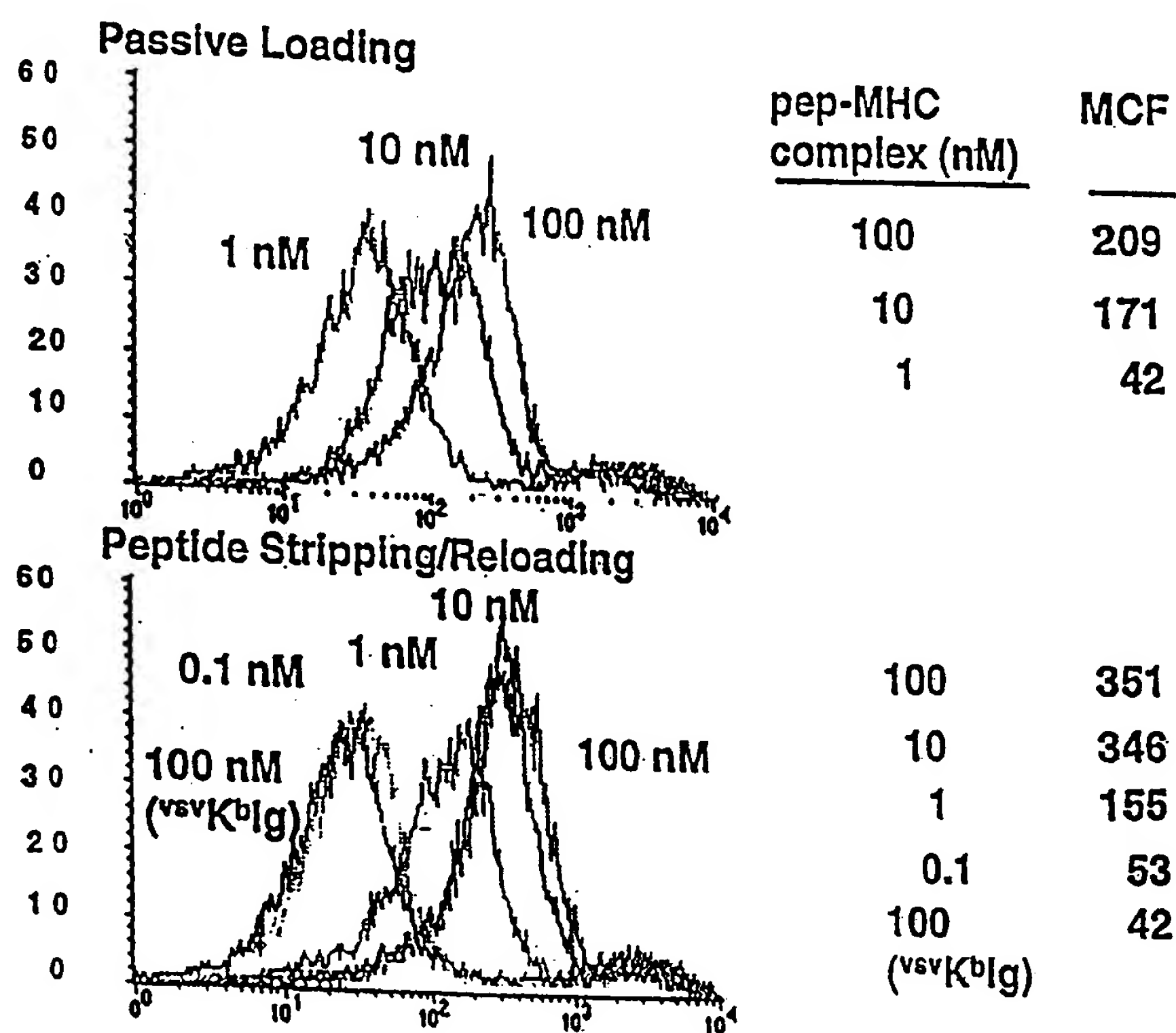


FIG. 5

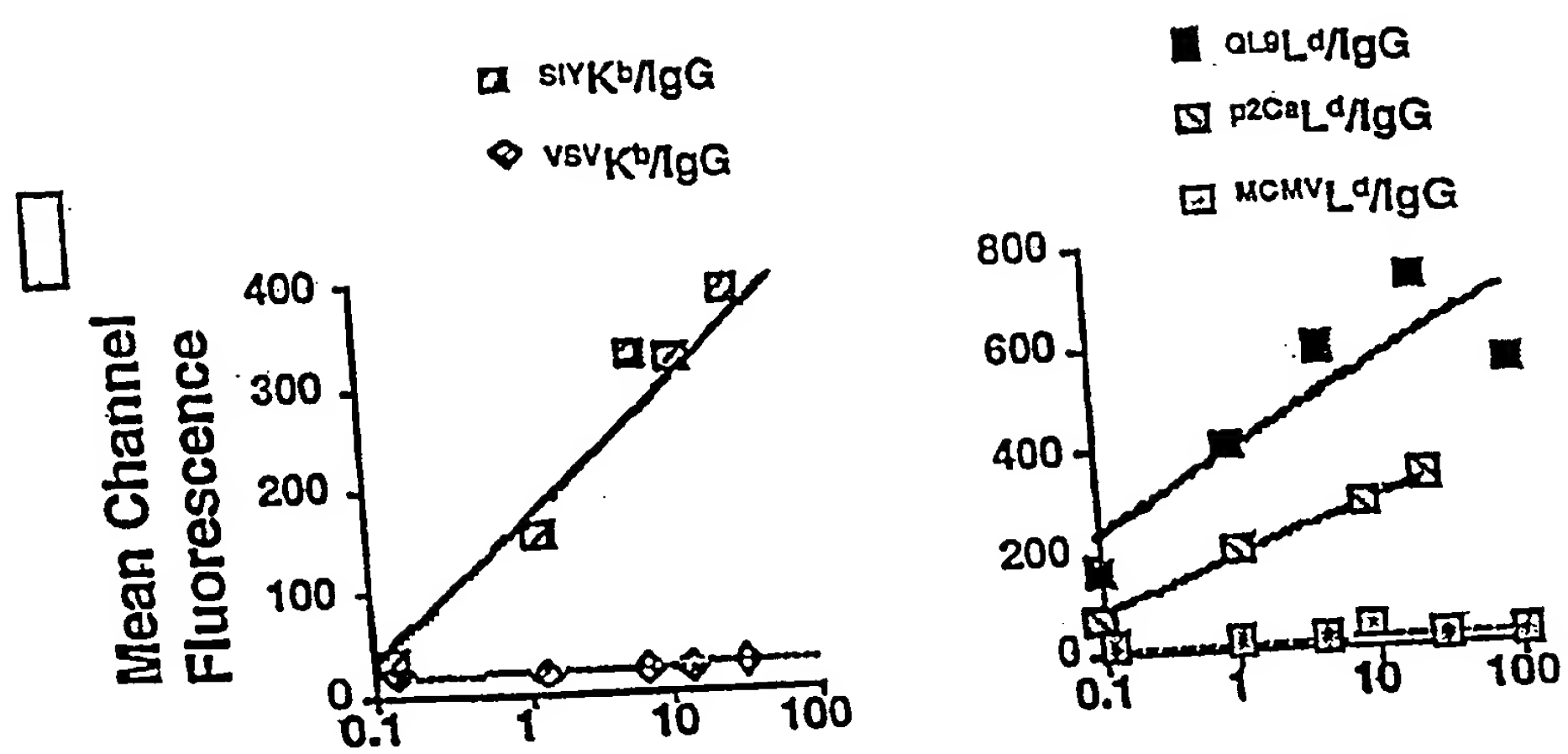


FIG. 6

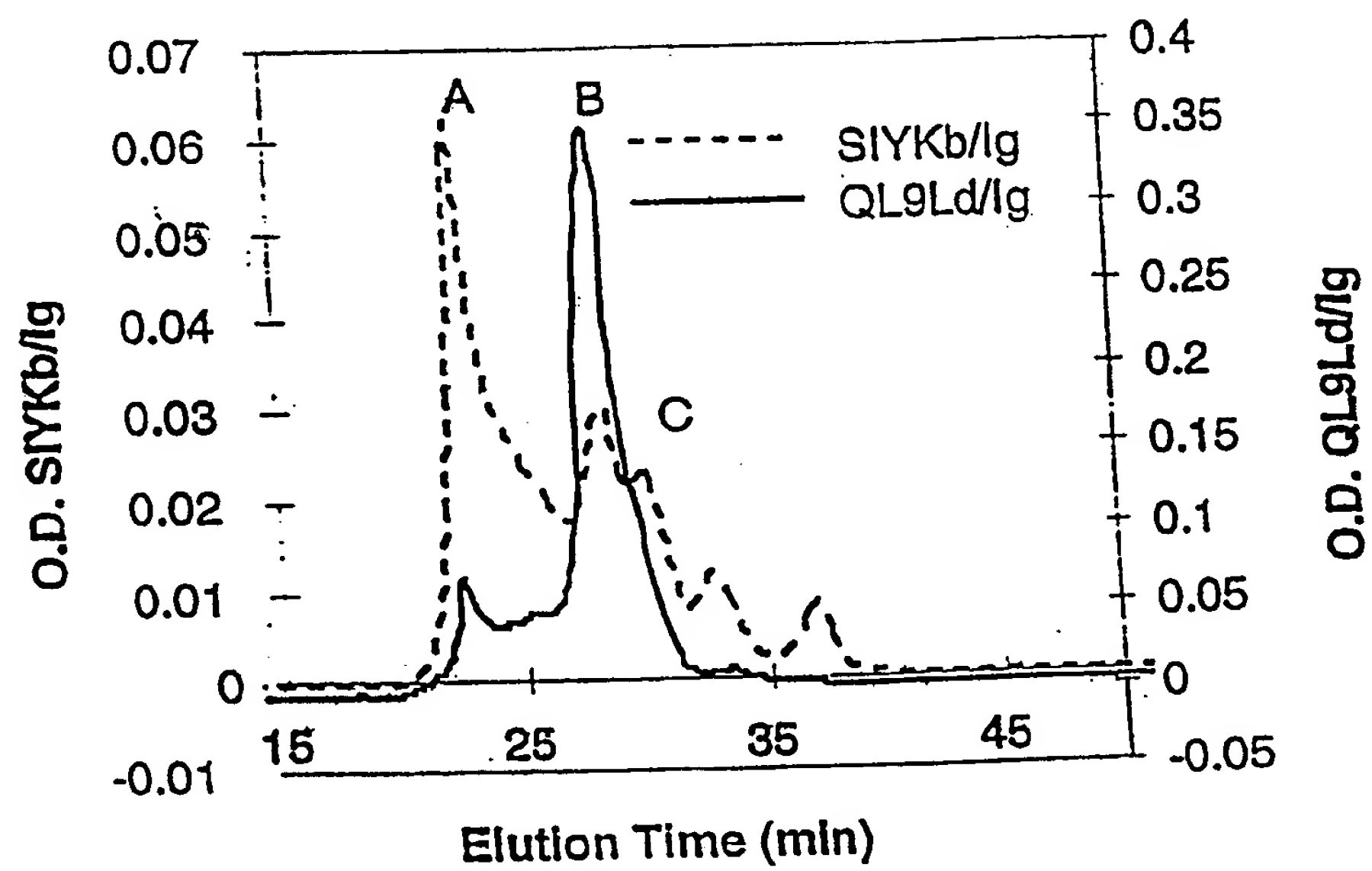


FIG. 7

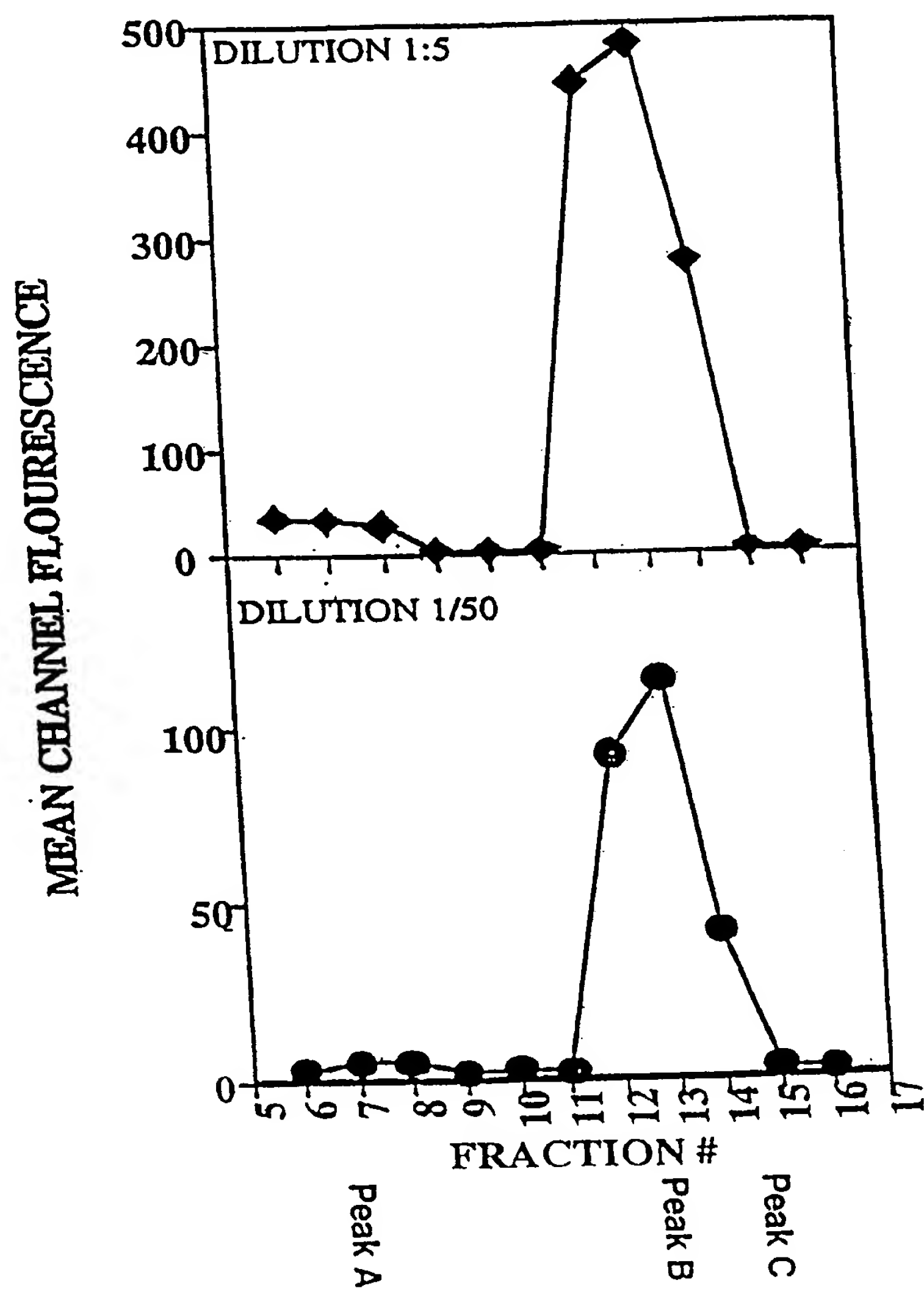
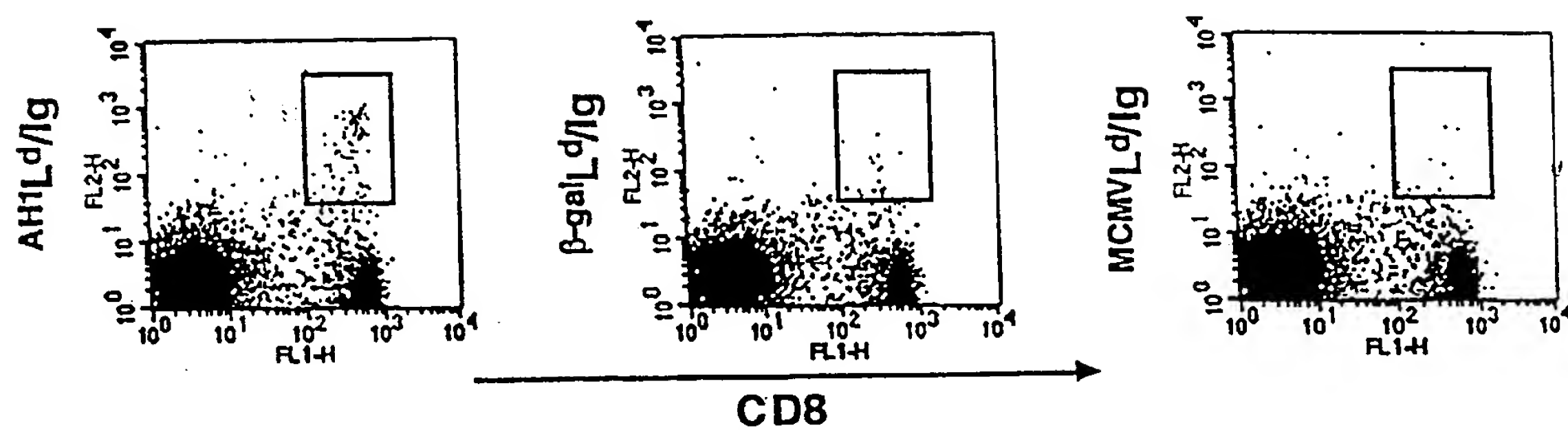
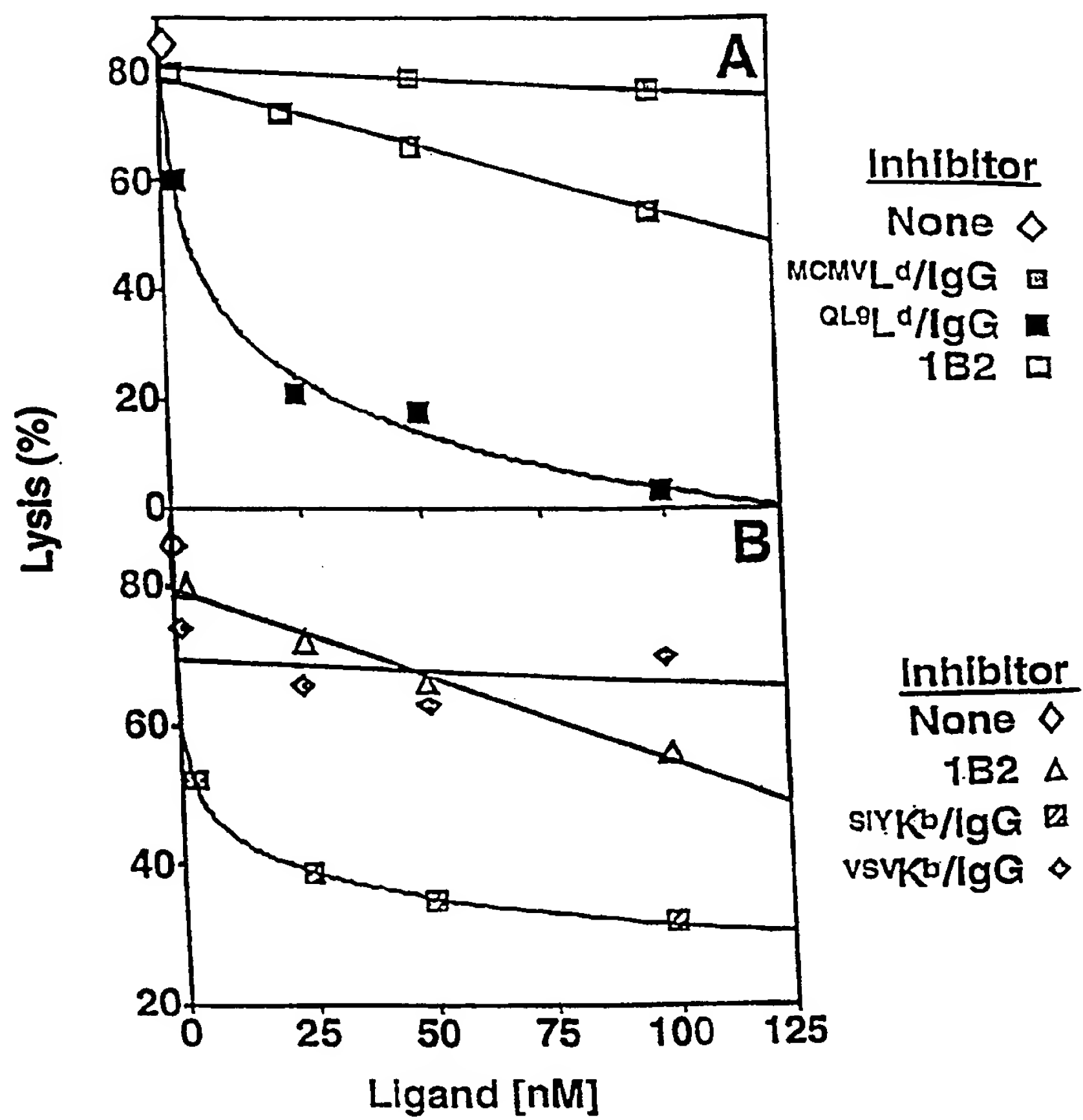


FIG. 8



<u>CT26 Immunized Animals</u>				<u>Naive Animals</u>			
	<u>Events</u>	<u>% Total</u>	<u>% CD 8</u>	<u>Events</u>	<u>% Total</u>	<u>% CD 8</u>	
AH1L ^d /Ig	640	0.32	3.0	69		0.32	
	649	0.32	3.3	109		0.54	
β-galL ^d /Ig	48	0.02	0.19	84		0.45	
	64	0.03	0.30	115		0.65	
MCMVL ^d /IgG	47	0.02	0.19	93		0.53	
	119	0.06	0.62	80		0.44	

FIG. 9



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FIG. 10

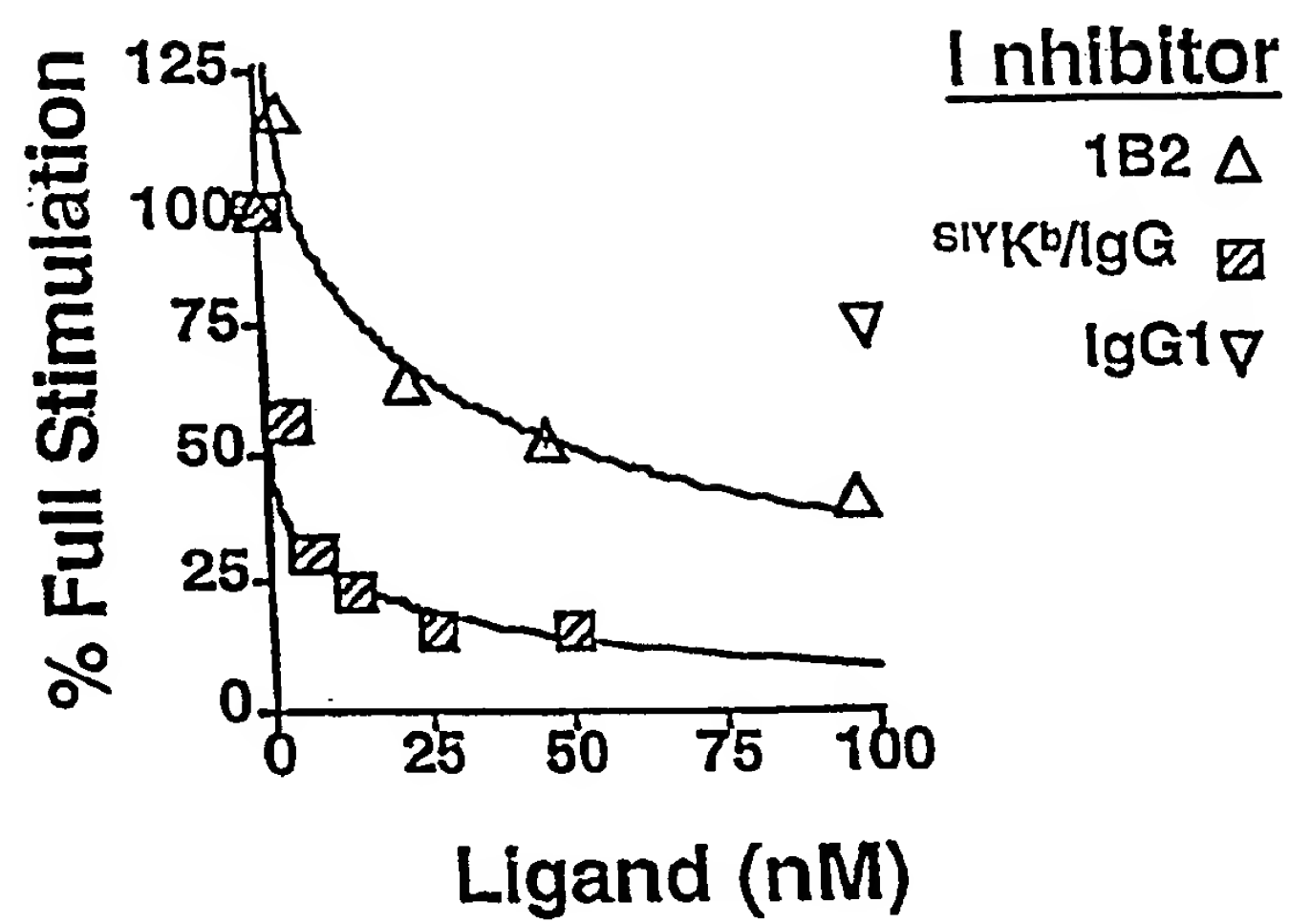


Figure 11A

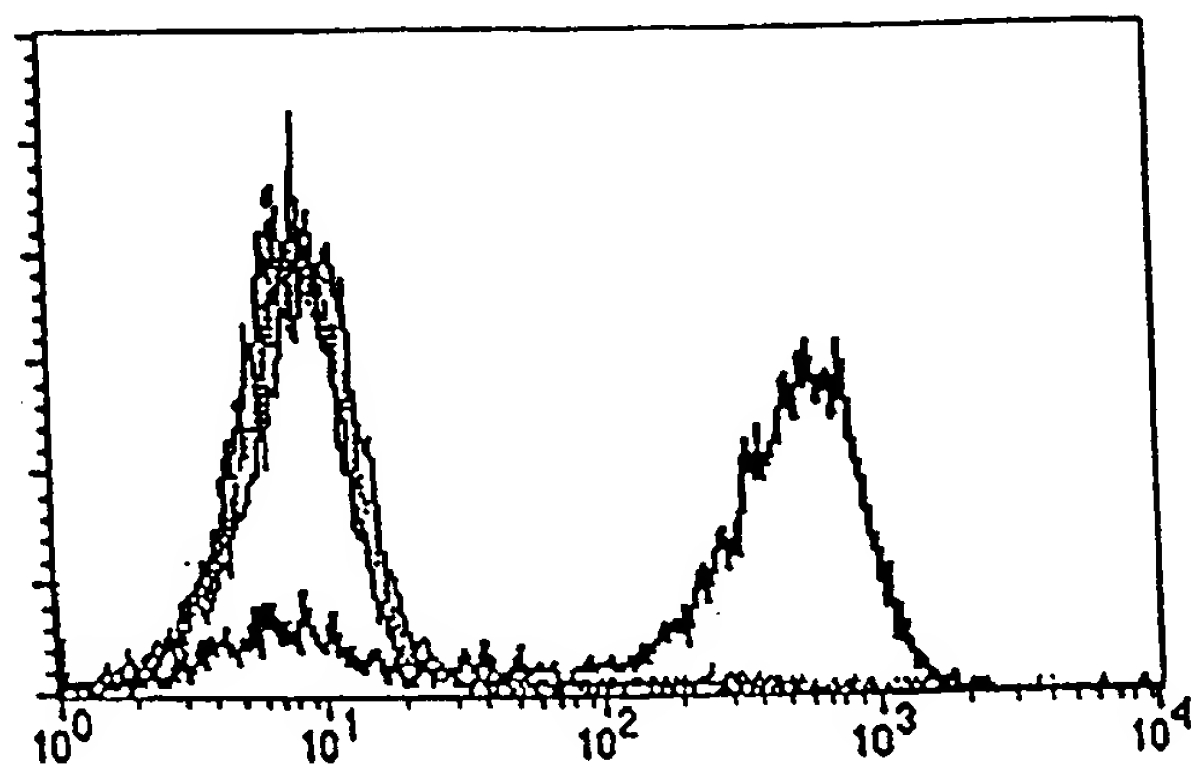
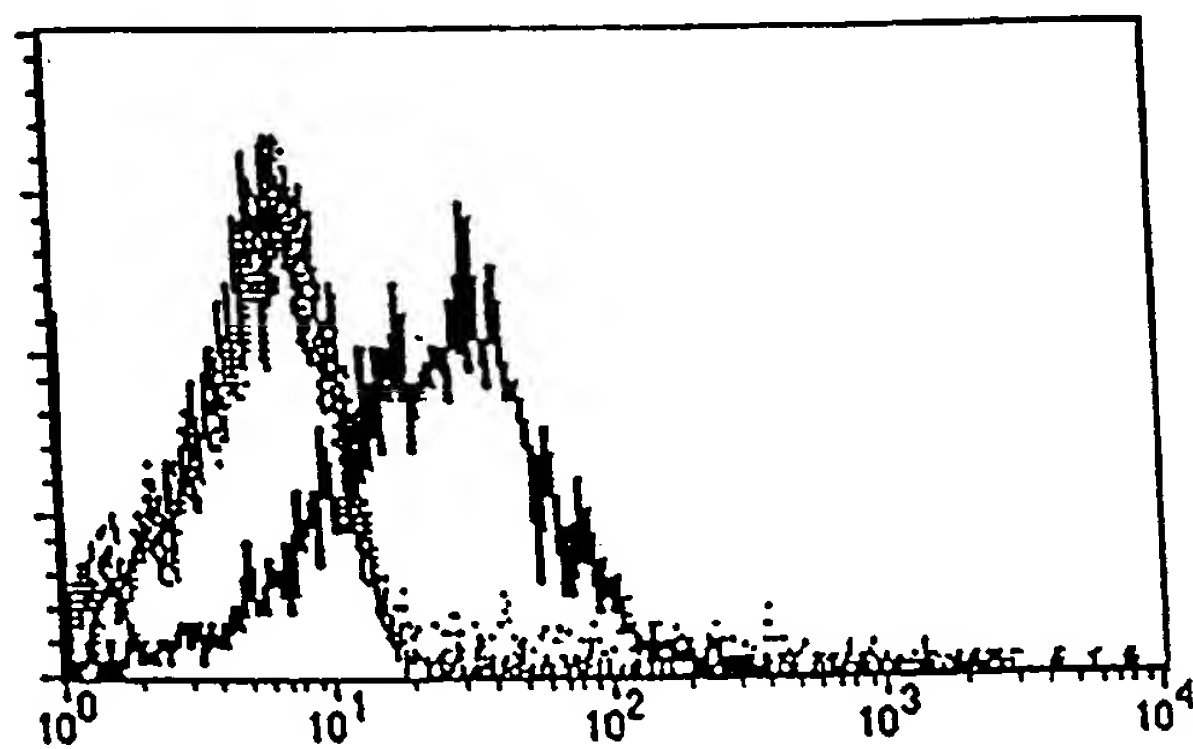


Figure 11B



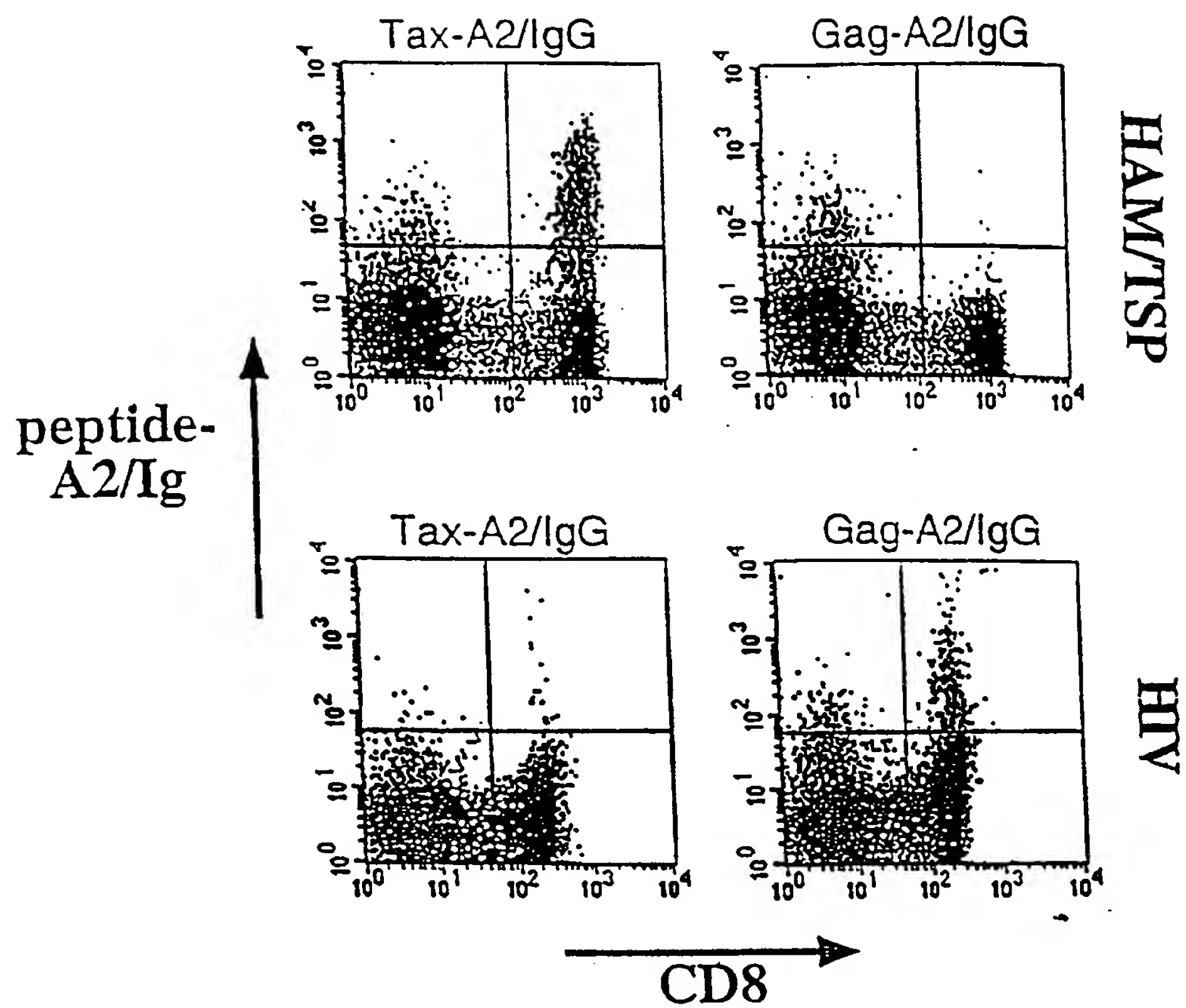


Figure 12

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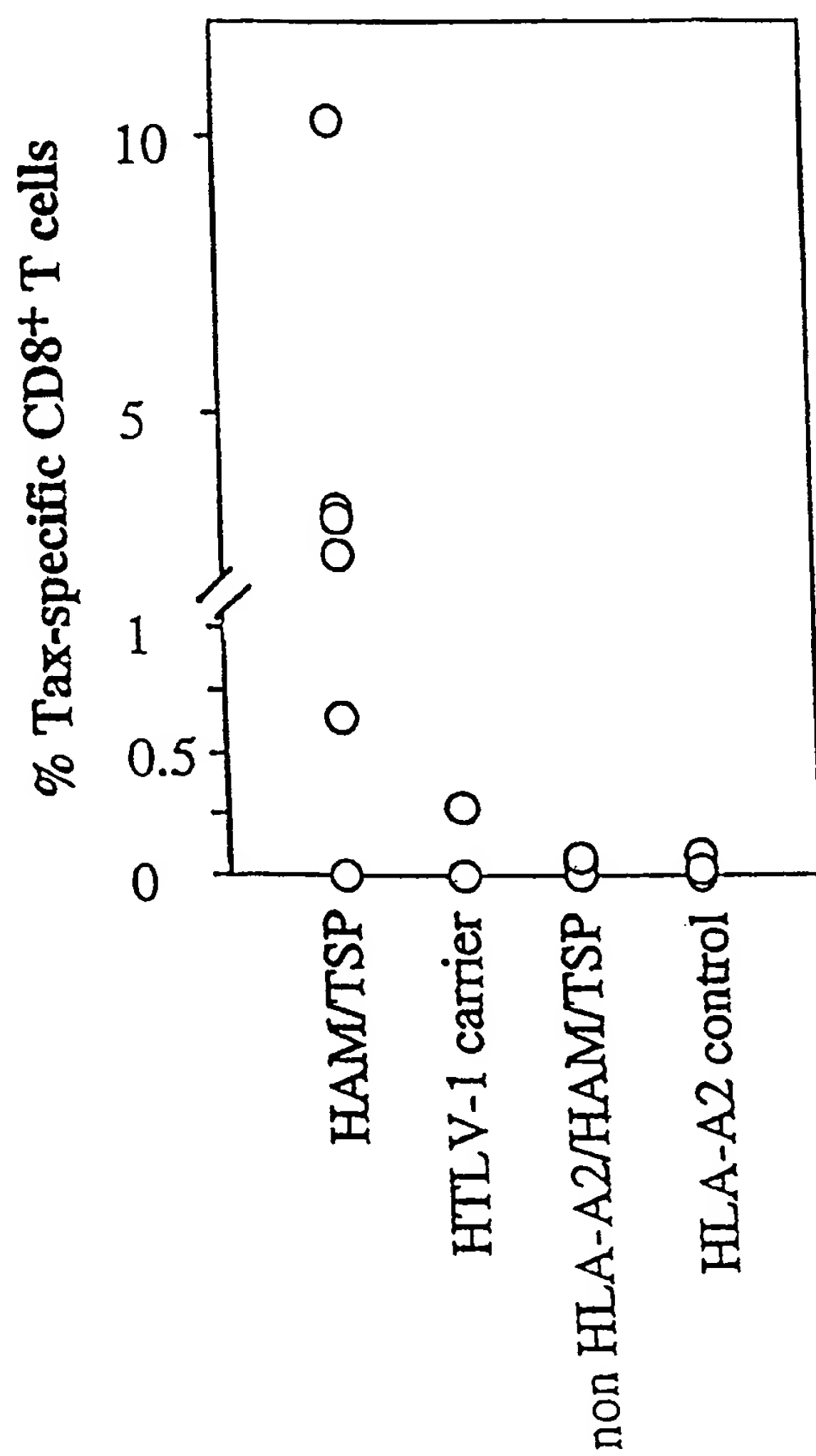


Figure 13

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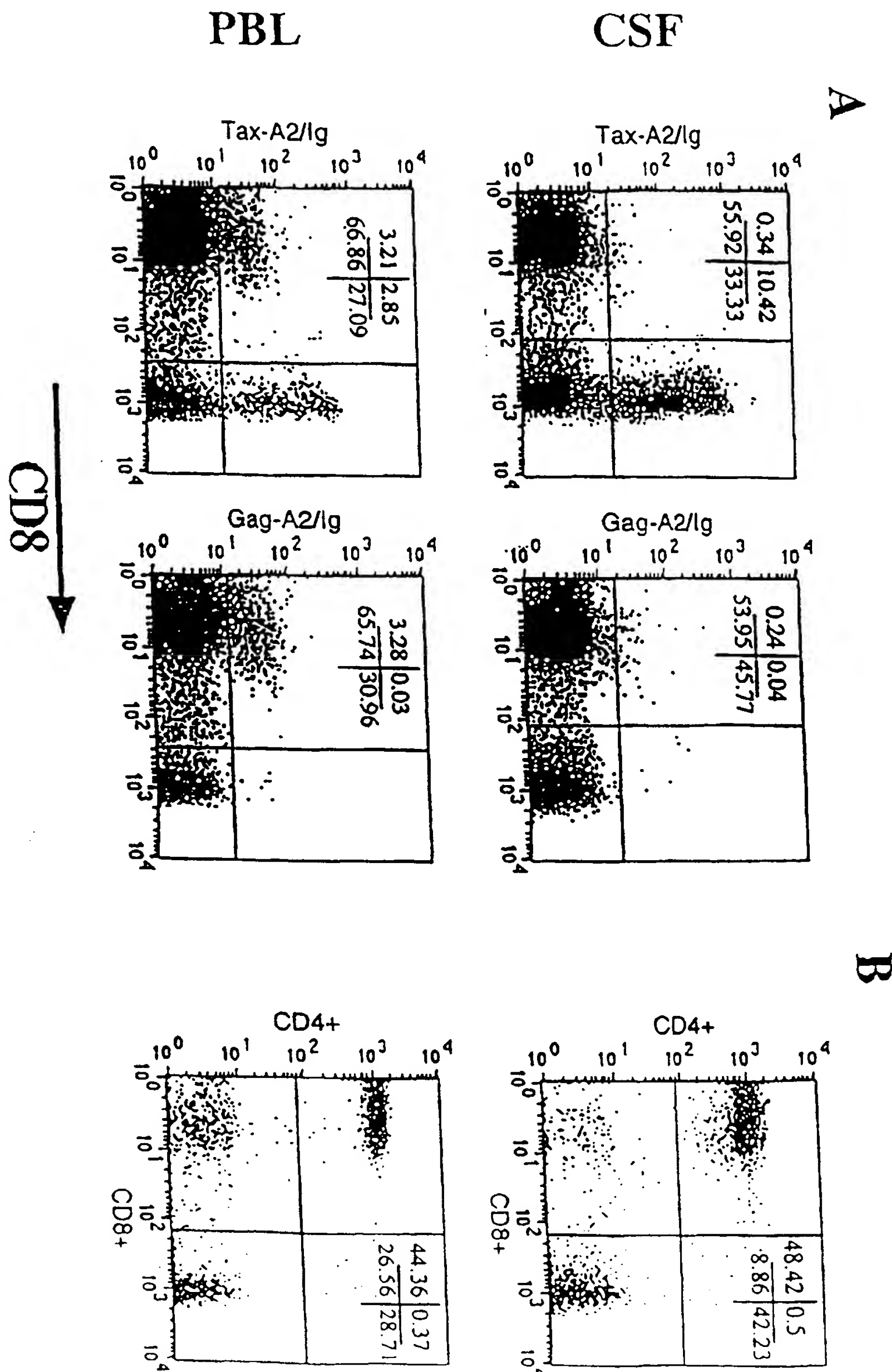


Figure 14

Fig. 15A

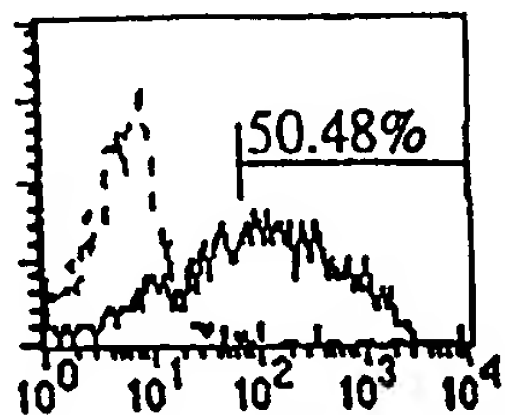


Fig. 15 B

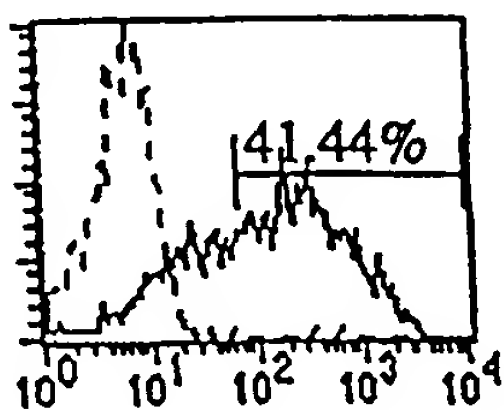


Fig. 15 D

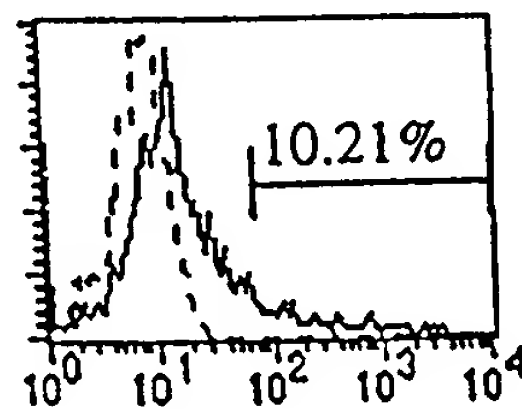


Fig. 15 C

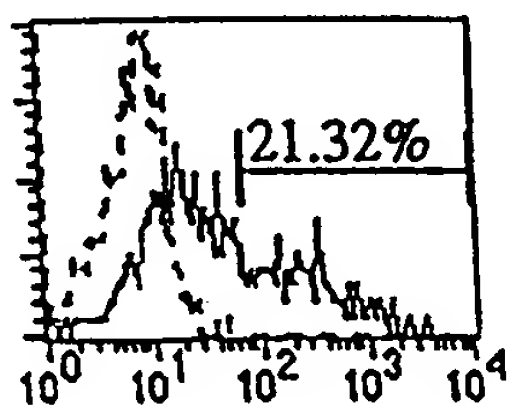
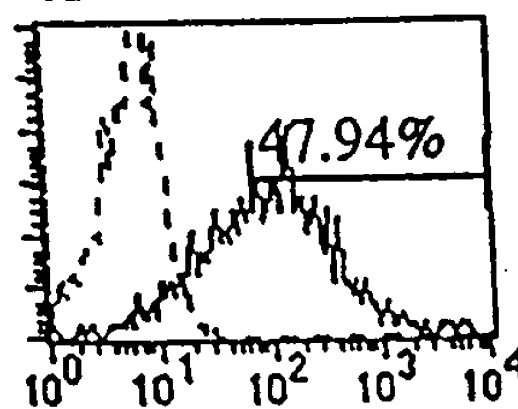
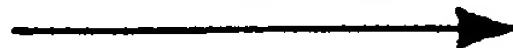


Fig. 15 E



HLA-DR



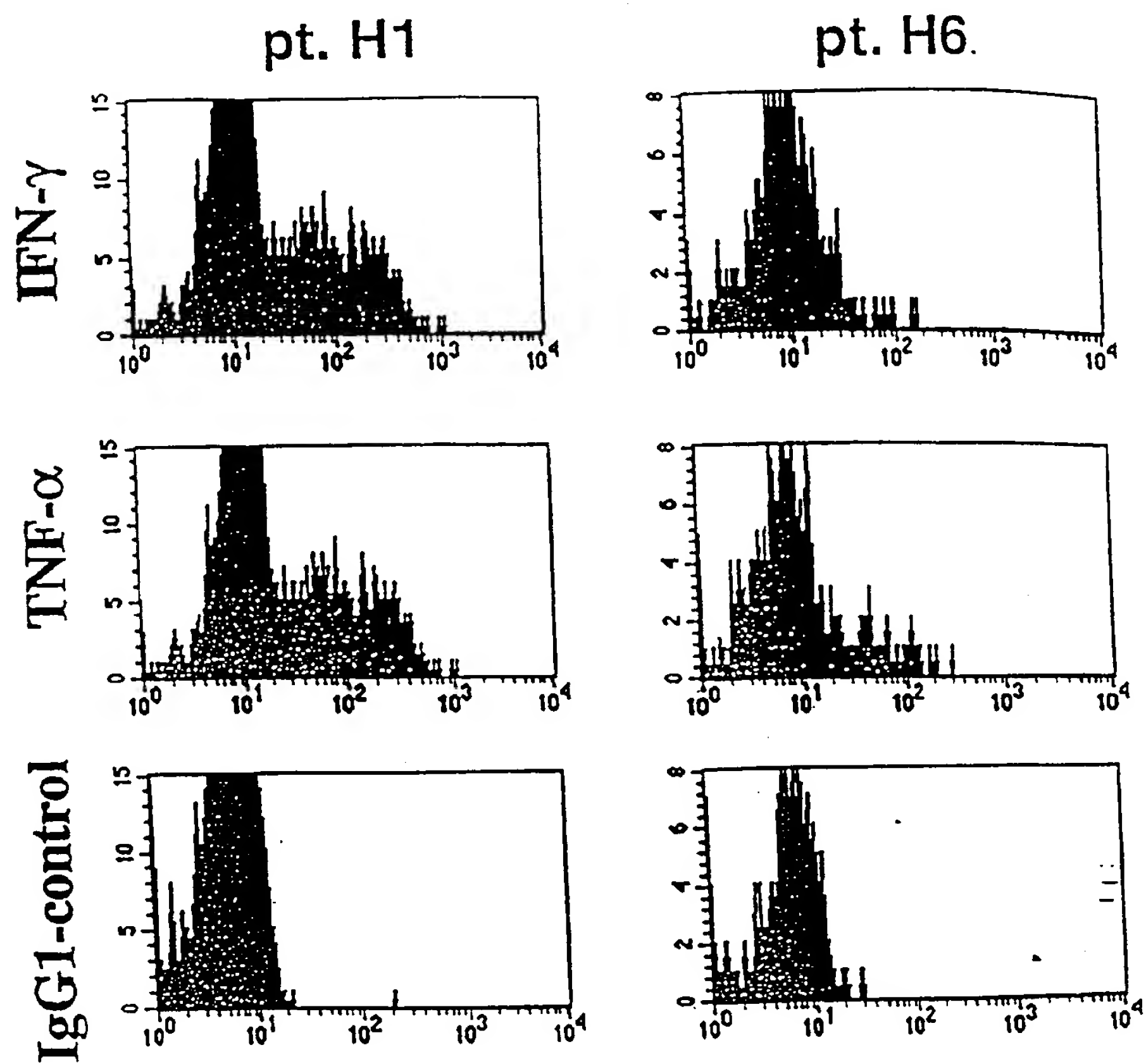


Figure 16

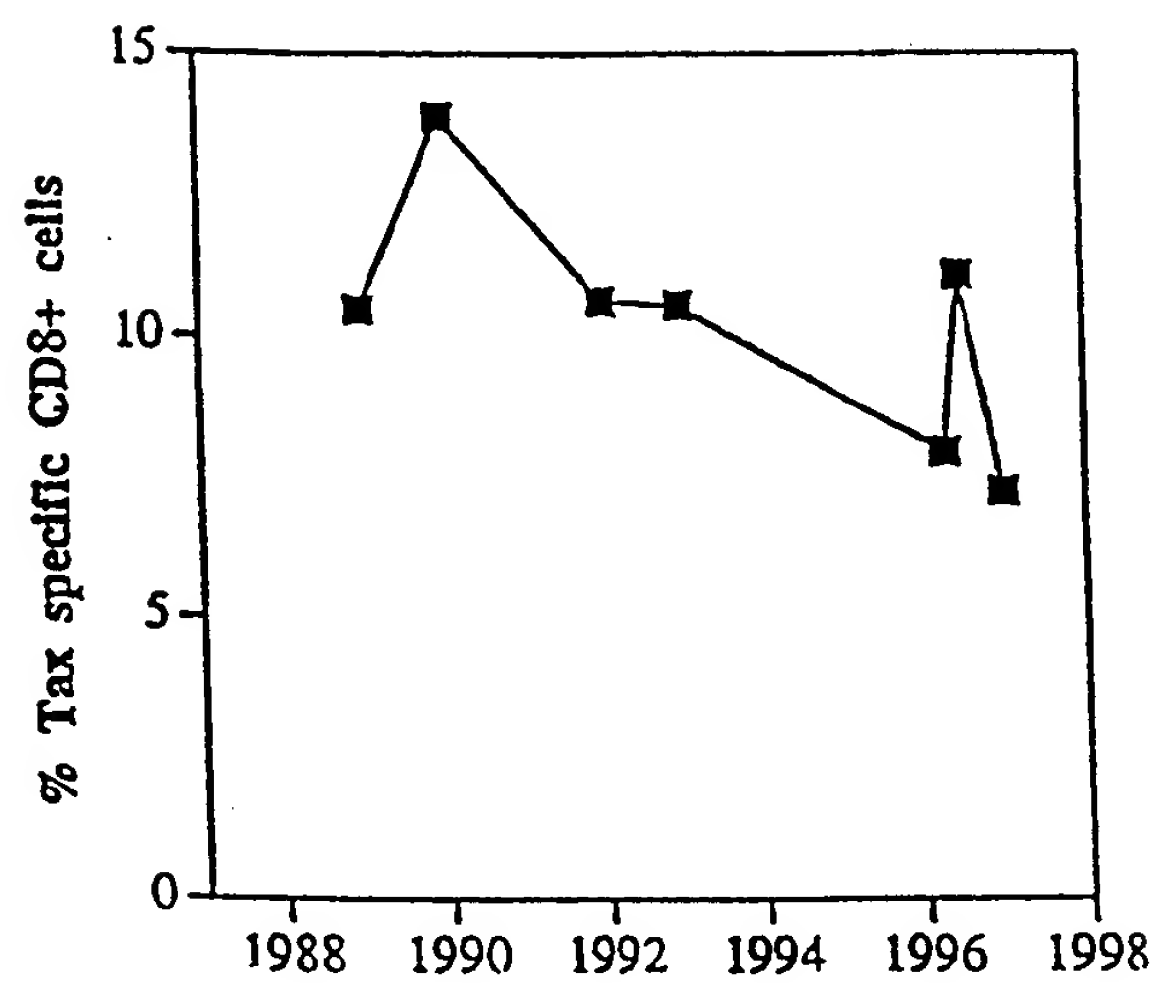


Figure 17

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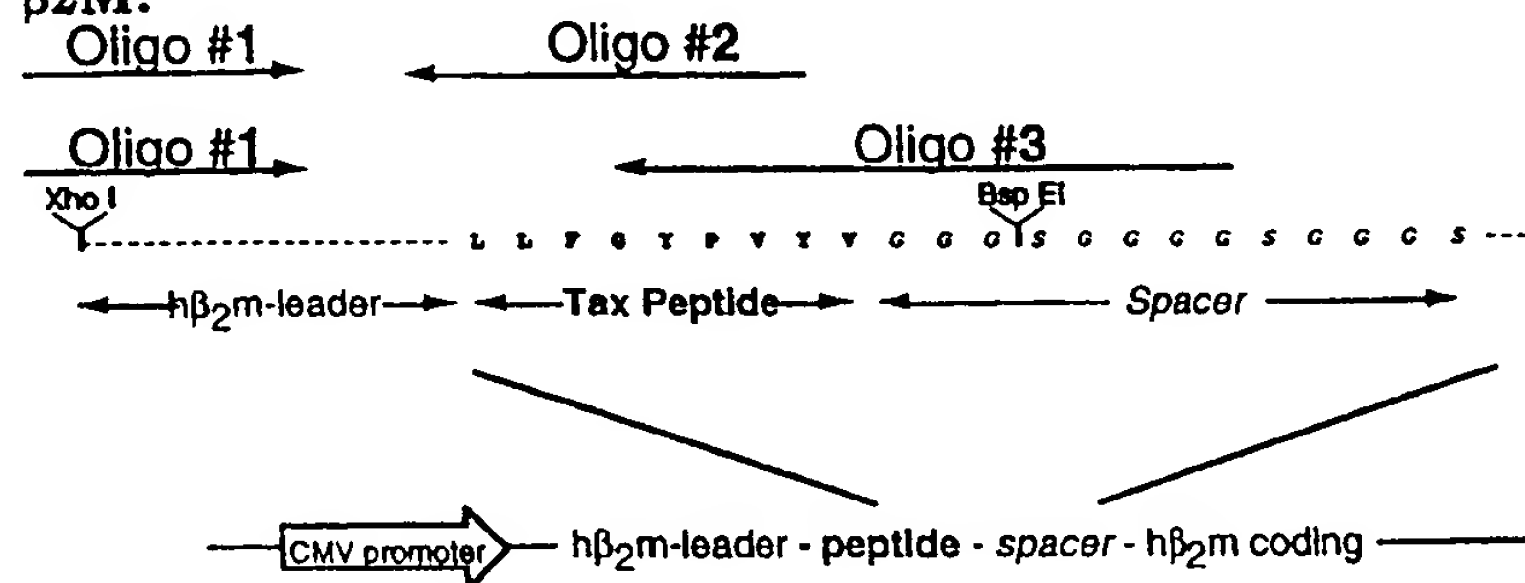
Figure 18: 3-D Structure of HLA-A2 Complexed With Tax 11-19 Peptide.

Legend: Two views of the 3-D structure of HLA-A2 heavy chain (green), β 2M (purple) and tax 11-19 antigenic peptide (red) are shown. The carboxyl terminal, position 9, of tax 11-19 peptide and amino terminal position 1 of the β 2M polypeptides are represented as space filling diagrams while the rest of the chains are shown as ribbon diagrams. Oxygen groups in the space filled amino acid residues are represented as red balls while the nitrogen groups are seen as light blue ones. The distance between the amino terminus of the β 2M molecule and position 9 of the peptide was found to be 20.49 Å. Open access of the region between



the ends of the peptide and β 2M molecule can be seen best on the side view of the complex (left hand side of Figure). Construction of the peptide tethered to β 2M will result in a decreased affinity of the peptide for the class I complex secondary to modification of the carboxyl terminal tax peptide, position 9. Anchoring of the peptide to the MHC molecule is achieved in part through bonds to the carboxyl terminal oxygen that will be unavailable in the tethered peptide- β 2M complex. This decreased affinity may be compensated for by stabilization with β 2M. Experiments underway with tax- β 2M constructs as described later in the proposal will directly address this issue.

Figure 19 Schematic Oligo design used to generate peptides-tethered to β_2M .



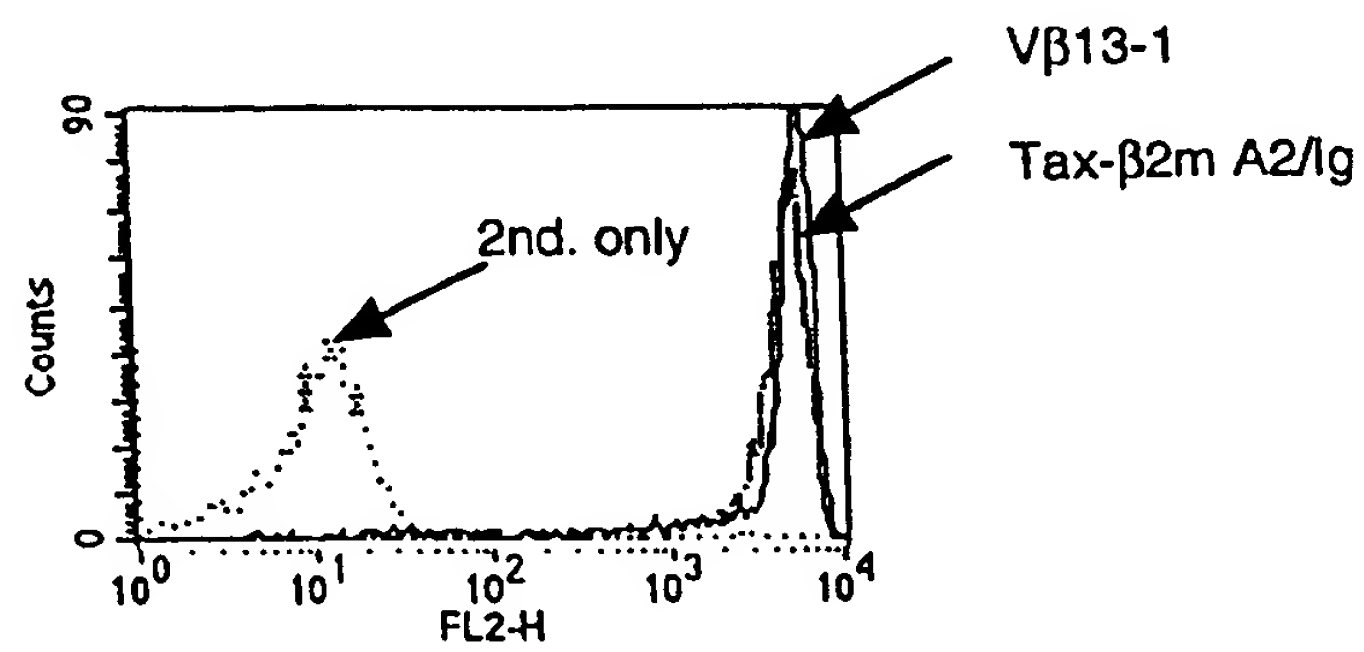


Figure 20.

SEQUENCE LISTING

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<212> DNA

<213> Homo sapiens

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<211> 36

<212> DNA

<213> Homo sapiens

<400> 5

cagtcgatgc ggccgcccat ctcagggtga ggggct

36



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁶ :</p> <p>C12N 15/85, A61K 38/17, 39/395, 47/48, 39/21, 39/145, 35/12, G01N 33/53, A61K 51/10, C07K 14/705 // (A61K 39/395, 39:145, 38:17) (A61K 39/395, 39:21, 38:17)</p>	<p>A3</p>	<p>(11) International Publication Number: WO 99/13095</p> <p>(43) International Publication Date: 18 March 1999 (18.03.99)</p>						
<p>(21) International Application Number: PCT/US98/18909</p> <p>(22) International Filing Date: 11 September 1998 (11.09.98)</p> <p>(30) Priority Data:</p> <table border="0"> <tr> <td>60/058,573</td> <td>11 September 1997 (11.09.97)</td> <td>US</td> </tr> <tr> <td>60/082,538</td> <td>21 April 1998 (21.04.98)</td> <td>US</td> </tr> </table> <p>(71) Applicant (for all designated States except US): THE JOHNS HOPKINS UNIVERSITY SCHOOL OF MEDICINE [US/US]; Suite 2-100, 2024 E. Monument Street, Baltimore, MD 21205 (US).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): SCHNECK, Jonathan [US/US]; Suite 2-100, 2024 E. Monument Street, Baltimore, MD 21205 (US). PARDOLL, Drew [US/US]; Suite 2-100, 2024 E. Monument Street, Baltimore, MD 21205 (US). O'HERRIN, Sean, M. [US/US]; Suite 2-100, 2024 E. Monument Street, Baltimore, MD 21205 (US). SLANSKY, Jill [US/US]; Suite 2-100, 2024 E. Monument Street, Baltimore, MD 21205 (US). GRETEN, Tim [US/US]; Suite 2-100, 2024 E. Monument Street, Baltimore, MD 21205 (US).</p>		60/058,573	11 September 1997 (11.09.97)	US	60/082,538	21 April 1998 (21.04.98)	US	<p>(74) Agents: KAGAN, Sarah, A. et al.; Banner & Witcoff, Ltd., 11th floor, 1001 G Street, N.W., Washington, DC 20001-4597 (US).</p> <p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report.</p> <p>(88) Date of publication of the international search report: 10 June 1999 (10.06.99)</p>
60/058,573	11 September 1997 (11.09.97)	US						
60/082,538	21 April 1998 (21.04.98)	US						
<p>(54) Title: USE OF MULTIVALENT CHIMERIC PEPTIDE-LOADED, MHC/IG MOLECULES TO DETECT, ACTIVATE OR SUPPRESS ANTIGEN-SPECIFIC T CELL-DEPENDENT IMMUNE RESPONSES</p> <p>(57) Abstract</p> <p>To increase the effective affinity of soluble analogs of peptide/MHC molecules for their cognate ligands, divalent peptide/MHC complexes were constructed. Using a recombinant DNA strategy, DNA encoding the MHC class I was ligated to DNA coding for murine Ig heavy chain. MHC/Ig complexes were exploited to homogeneously load with peptides of interest. The results of flow cytometry demonstrated that the ^{Pc}PMHC/Ig complexes bound specifically with high affinity to cells bearing their cognate receptors. ^{Pc}PMHC/Ig complexes are also useful in modulating effector functions of antigen-specific T cells. These ^{Pc}PMHC/Ig complexes are useful for studying TCR/MHC interactions and lymphocyte tracking and have uses as specific regulators of immune responses.</p> <div data-bbox="1092 1542 1856 2227" style="text-align: center;"> </div>								

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INTERNATIONAL SEARCH REPORT

Int. tional Application No
PCT/US 98/18909

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/85 A61K38/17 A61K39/395 A61K47/48 A61K39/21
A61K39/145 A61K35/12 G01N33/53 A61K51/10 C07K14/705
/(A61K39/395, 39:145, 38:17), (A61K39/395, 39:21, 38:17)

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

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Y	see the whole document	14, 27, 31, 34, 35, 39, 40, 50
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Patent family members are listed in annex.

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Date of the actual completion of the international search

18 March 1999

Date of mailing of the international search report

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Authorized officer

Hagenmaier, S

INTERNATIONAL SEARCH REPORT

Int. Application No.

PCT/US 98/18909

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X	SCHNECK J P ET AL: "SPECIFIC INHIBITION OF GRAFT REJECTION BY SOLUBLE MHC SUPERDIMERS" FASEB JOURNAL, vol. 10, no. 6, 30 April 1996, page A1473 XP002039184	1-5, 9-13, 15-26, 28-30, 32,33, 36-38, 41-49, 51,52
Y	see the whole document	14,27, 31,34, 35,39, 40,50
X	--- PORTO DAL J ET AL: "A SOLUBLE DIVALENT CLASS I MAJOR HISTOCOMPATIBILITY COMPLEX MOLECULE INHIBITS ALLOREACTIVE T CELLS AT NANOMOLAR CONCENTRATIONS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 90, no. 14, 15 July 1993, pages 6671-6675, XP002039180	1-5, 9-13, 15-26, 28-30, 32,33, 36-38, 41-49, 51,52
Y	see the whole document	14,27, 31,34, 35,39, 40,50
X	--- JOHANSEN T E ET AL: "POTENT INHIBITION OF ALLOREACTIVE T CELLS BY NANOMOLAR CONCENTRATIONS OF A DIVALENT SOLUBLE CLASS I MHC MOLECULE" JOURNAL OF IMMUNOLOGY, vol. 150, no. 8, PART 02, 15 April 1993, page 83A XP002039181	1-5, 9-13, 15-26, 28-30, 32,33, 36-38, 41-49, 51,52
Y	see the whole document	14,27, 31,34, 35,39, 40,50
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Y	see the whole document	14,27, 31,34, 35,39,40
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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/18909

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>WO 94 19473 A (SOMATOGEN INC) 1 September 1994 Example 1, column 41-42 & DATABASE GCG_GENESEQ_P ID/AC: R61275, ANDERSON ET AL.: "Transactivating protein, Tax, peptide fragment #1" Peptide with 100% identity in 9 aa overlap with Seq.ID 1. see abstract</p>	14,27, 31,50
Y	<p>US 5 420 244 A (LAL RENU B ET AL) 30 May 1995 Example 1, columns 11-12 & DATABASE GCG_GENESEQ_P ID/AC: R74356, LAL: "HTLV-1 p40 (tax) protein antigenic peptide Tax1" peptide with 100% identity in 9 aa overlap with Seq.ID 1. see abstract</p>	14,27, 31,50
Y	<p>WO 94 26903 A (UNIV LEIDEN ;SEED CAPITAL INVESTMENTS (NL); MELIEF CORNELIS JOSEPH) 24 November 1994 Claim 4, page 29 & DATABASE GCG_GENESEQ_P ID/AC: R66539, KAST ET AL.: "Human influenza virus M protein residues 58-68" Peptide with 100% identity in 9 aa overlap with Seq. ID 2. see abstract</p>	14,39, 40,50
Y	<p>WO 94 28871 A (ENDOCON INC) 22 December 1994 Disclosure, page 11 & DATABASE GCG_GENESEQ_P ID/AC: R68765, LEONARD: "Cytotoxic T lymphocyte epitope 22 derived from gag p17 protein" Peptide with 100% identity with Seq. ID 3 see abstract</p>	14,34, 35,50
A	<p>WO 96 20215 A (OM LAB SA ;DEUTSCHE OM ARZNEIMITTEL GMBH (DE); LAUENER ROGER PASCA) 4 July 1996 see the whole document</p>	1-52
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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/18909

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO 97 35991 A (UNIV JOHNS HOPKINS ;SCHNECK JONATHAN P (US); HERRIN SEAN O (US)) 2 October 1997 see the whole document ----	1-13, 15-26, 28-30, 32, 33, 36-38, 41-49, 51, 52
P, X	CASARES ET AL.: "ENGINEERING AND CHARACTERIZATION OF A MURINE MHC CLASS II-IMMUNOGLOBULIN CHIMERA EXPRESSING AN IMMUNODOMINANT CD4 T VIRAL EPITOPE" PROTEIN ENGINEERING, vol. 10, no. 11, November 1997, pages 1295-1301, XP002097124 see the whole document -----	1-10, 15, 19-26, 32, 33, 39, 41-46, 51, 52

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/18909

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 24-40
is(are) directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/18909

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